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ACTIVATED COMPLEMENT IN THE SPUTUM FROM PATIENTS WITH CYSTIC FIBROSIS

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Schiøtz P O Sørensen H & Høiby N Activated complement in the sputum from patients with cystic fibrosis Acta path microbiol scand Sect. C 87 1–5 1979

14 cystic fibrosis (CF) patients chronically infected with mucoid *P aeruginosa* and presenting multiple precipitins in serum against this bacterium (CF + P) and 13 CF patients without *P aeruginosa* infection (CF P) had their plasma and sputum sol phase examined for albumin C1q C3/C3c C4 and C5 by means of electroimmunoassays Their sputum sol phase was examined also for factor B by rocketimmunoelectrophoresis C3c was demonstrated in the sputum sol phase but significantly more frequent ($p < 0.01$) among the CF + P patients than among the CF P patients Factor $\bar{B}$ was also demonstrated in the sputum sol phase but no significant difference in frequency could be demonstrated between the CF + P and the CF P patients None of the results indicated that a local pulmonary production of complement factors took place Complement activation was significantly ($p < 0.01$) associated with inflammation expressed as increased

$$\frac{\text{sputum sol phase albumin}}{\text{plasma albumin}} \text{ ratio}$$

The results show the importance of complement mediated inflammation in the patogenesis of pulmonary tissue damage in patients with CF and support the concept of chronic *P aeruginosa* lung infection as an immune complex disease in CF patients

Key words Cystic fibrosis sputum complement *Pseudomonas aeruginosa*

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Cystic fibrosis (CF) patients suffer from chronic and recurrent bacterial lung infections and especially chronic *P aeruginosa* lung infection has been shown to be associated with a pronounced humoral [illegible]

significantly more frequent in patients with chronic *P aeruginosa* lung infection than in patients [illegible]

factors were present in sputum from CF patients as part of their local pulmonary immune defense mechanisms Furthermore activated complement factors promote the development of inflammation (11) so we examined whether an association between chronic *P aeruginosa* lung infection and complement activation could be demonstrated

PATIENTS AND METHODS

Patients

Twenty-seven CF patients were included in the study All patients had a typical history of CF and marked elevated sweat electrolytes in repeated tests (4)

(i) One group consisted of 14 CF patients (6 females and 8 males, median age 13 9 years, range 6–29) suffering from chronic infection with mucoid strains of *P aeruginosa* (CF+P) and exhibiting more than 10 different precipitins in serum against watersoluble antigens from this bacterium

The patients have been followed as previously described (7) Mean duration of the *P aeruginosa* respiratory tract infection was 4 1 years (range 1–8 5 years) Routine studies of the lung function showed a reduced vital capacity and peak expiratory flow rate as compared with normal values (19) On an average, the vital capacity of the lungs was 2 1 SD below mean normal values, and the peak expiratory flow rate was 1 6 SD below normal values in healthy persons of the same height

(ii) Thirteen CF patients (6 females and 7 males, median age 13 8 years, range 8–21) without *P aeruginosa infection (CF-P) and without multiple serum* precipitins against *P aeruginosa* antigens were examined also The average vital capacity of their lungs was 1 SD below mean normal values, and the peak expiratory flow rate was 0 3 SD above mean normal values (19) In 9 of the CF-P patients *S aureus H influenzae, E coli S pneumoniae B anitratum* and *K ozanae* were isolated from the respiratory tract during this study

Sputum

A 3-hours sample of sputum was collected at 4°C from each patient between 8 a m and 11 a m as described previously (1, 14) Each sample was subjected to bacteriological examination and the origin of the specimens from the lower respiratory tract was confirmed by studying the epithelial cells present (7) Sol phase of sputum was obtained by ultracentrifugation at 120,000 × *g* (maximum value) at 4°C for 4 hours and stored in small aliquots at −80°C (14)

P aeruginosa *Precipitins*

The occurrence of circulating precipitating antibodies in serum against *P aeruginosa* were investigated by means of crossed immunoelectrophoresis (microtechnique) as described previously (7)

Complement Factors and Albumin

Samples of venous blood stabilized with EDTA and centrifuged were drawn from all patients The plasma was stored in small aliquots in liquid nitrogen at −192°C until analyzed

Albumin and complement components C1q C3 C4 and C5 were determined in plasma and sputum sol phase by means of routine electroimmunoassays (10) The results of the C1q C4 and C5 determinations were expressed in arbitrary units as a percentage of a reference standard consisting of pooled EDTA plasma obtained from 100 healthy blood donors The sensitivity of the analyses for C1q C4 and C5 was 10 5 and 5 arbitrary units respectively For C3 Standard Human Serum (Behringwerke Marburg West Germany) was used as reference The sensitivity of the analysis was 0 05 g/l

Conversion of C3 was detected by crossed immunoelectrophoresis of 10 μl sputum sol phase performed as high voltage electrophoresis at 18 V/cm for 2½ hours in the first dimension The second dimension electrophoresis was performed at 3V/cm for 18 hours into an agarose gel containing monospecific antiserum against C3 (β1C/β1A, from Dakopatts, Copenhagen) (concentration 5 μl/cm²) The dried gel slides were stained with Coomassie brilliant blue and investigated for C3 and C3 split products (18) Exact quantitative determination of the C3 split products was not performed Control ultracentrifugation of serum at 4°C showed no activation of complement caused by this procedure

The presence of factor B in sputum sol phase was determined by rocketimmunoelectrophoresis of 3 μl sputum sol phase in agarose containing specific factor B antiserum (Behringwerke, Marburg, West Germany) (concentrations 3 μl/cm²) With a suitable buffer system (barbitone/calciumlactate buffer, pH 8 6, ionic strength of vessel buffer 0 065 and of gel buffer 0 025) and by employing 1% agarose (Litex type HSA, Glostrup, Denmark) factor $\bar{B}$ was revealed as a reverse rocket which migrated towards the cathode while native factor B migrated towards the anode Exact quantitation of the activated and native form of factor B was not performed

Statistical Methods

The Mann-Whitney test and Fisher s test were used and a significance level of 5% (double tailed test) was chosen (2)

RESULTS

The plasma C1q C3, C4 and C5 values from the CF+P patients did not differ significantly from those from the CF-P patients

Sputum sol phase examinations for C1q were all negative Complement C3, C4 and C5 was present in sputum sol phase in *13, 9 and 6 patients* respectively All the values were so low, that it was only possible to give a qualitative result present or not present (Table 1)

C4 was found in the sputum sol phase in 8 patients in the CF+P group, while one patient in the CF-P group was positive for C4 This difference is significant ($p < 0.02$) (Table 1) The same comparison for C5 in the sputum sol phase (5 patients in the CF+P group versus 1 patient in the CF-P group) showed no significant difference between the 2 groups ($p > 0.05$) (Table 1)

Examination for conversion of C3 in the sputum sol phase showed that C3 was present solely as C3c and no native C3 in the 13 patients where C3 could be demonstrated by rocket immunoelectrophoresis (Fig 1) None of these patients had C3c in their plasma and no other patients had C3c neither in their sputum sol phase nor in plasma 11 of the patients with the *C3 split product C3c were from* the CF+P group and 2 were from the CF-P group

TABLE I *Albumin Ratios* ($\frac{\text{Sol Phase Albumin Conc}}{\text{Plasma Albumin Conc}}$ *× 100 %) and Occurrence of C3c, C4 C5 and Factor B in Sputum Sol Phase from Cystic Fibrosis Patients with (CF + P) and without (CF-P)* Pseudomonas aeruginosa *Infection*

Patient	no	Albumin ratio (%)	C3c	C4	C5	Factor B/B̃
CF + P	1	0 6	+			
	2	0 7				+
	3	1 0	+			+
	4	5 1	+	+		+
	5	8 9	+	+	+	+
	6	11 4	+	+	+	+
	7	6 2	+	+	+	+
	8	5 9	+	+	+	+
	9	3 5	+	+		
	10	0 4		+		+
	11	2 3	+	+		+
	12	2 3			+	+
	13	1 3	+			+
	14	1 5	+			
CF P	15	0 7				+
	16	1 2				
	17	0 7				
	18	2 6	+	+	+	+
	19	0 7				
	20	5 7				
	21	1 3				+
	22	2 8	+			+
	23	0 4				
	24	0 4				
	25	2 3				
	26	0 9				+
	27	1 3				
Significance of difference		n s	$p<0\,01$	$p<0\,02$	n s	n s

\+ = presence of complement component but the concentration too low to *permit exact quantitation*

(Table 1) This difference is significant ($p<0\,01$)

Examination for factor B was positive in the sputum sol phase in 16 patients and all these patients showed activation of factor B as well (Table 1) 11 of these patients were from the CF + P group and 5 were from the CF-P group this difference is not *significant* ($p = 0\,10$)

An estimate of transudation of *serum proteins* to sputum caused by inflammation was obtained by calculating [illegible]

in the CF-P group showed no significant difference between the groups ($p>0\,05$) (Table 1) A comparison of albumin ratios between the 13 patients with the C3 split product C3c and the remaining other 14 patients showed however a significant higher *ratio* in the *former group* ($p<0\,01$), on an average 4 1 % versus 1 4 % A *comparison* of the same albumin ratios between the 16 patients with native and activated factor B in their sputum sol phase and the 11 patients *without signs of factor* B in their sputum sol phase was also significant ($p<0\,01$) on an average the ratios were 3 6 % versus 1 3 %

By multiplying the *plasma complement* concentrations with the albumin ratio an estimated sputum *sol phase* complement concentration was obtained, which may be explained as *transudation* of serum complement factors (ignoring the differences in molecular size between albumin and the complement factors) Compared to the analytical precision of the C1q C3, C4 and C5 electroimmunoassays all the *estimated sputum* sol phase complement values

were in agreement with the actual findings and the sputum sol phase values of C1q, C3, C4 and C5 were thus below 10 5, 5 and 5% respectively of the actual plasma values




Fig 1 Crossed immunoelectrophoresis of 2 samples into an agarose gel containing antiserum against C3 (β1A/β1C) (5 µl/cm²) Anode to the right

Above 10 µl heparin plasma from a normal person with immune complexes added (Human antialbumin/human serum albumin) showing conversion of C3 to C3c

Below 10 µl sputum sol phase from a cystic fibrosis patient showing solely the presence of C3c and no native C3

DISCUSSION

Complement components in bronchial secretions have been demonstrated by *Reynolds et al* (12) who found C4 and C6 in bronchial lavage fluid from smokers and non smokers in concentrations of about 3% of the serum values Similar values were demonstrated for C3 in sputum sol phase from CF patients by *Brogan et al* (1) These authors concluded that the complement concentrations probably were a result of passive transudation and these results are in accordance with our view

Furthermore evidence of local complement activation in the lungs notably from CF + P patients is presented in the present study As activation of C3 is a proteolysis caused by the C$\overline{T24}$ complex it cannot be ruled out that proteolytic enzymes liberated from e g neutrophils in the bronchial secretions may participate in the splitting of C3 (11)

The complement split product C3c was however found significantly more *frequent in the sputum sol phase among the CF + P* patients than among the CF-P patients although the latter group of patients *also harboured* bacteria in the lungs As soluble immune complexes have recently been demonstrated in the sputum sol phase in CF + P patients (16) it seems more likely that it is these complexes which activate complement (either via the classical or alternative pathway) and thus initiates the inflammatory process This is well in agreement with the conception of chronic *P aeruginosa* lung infection in CF as an immune complex disease associated with a poor prognosis (9, 16) The possibility however, still exists, that a potent alternative pathway activation (caused by bacterial products) is responsible for both factor $\overline{B}$ and C3c in the sputum sol phase (3, 5, 17)

The significantly higher albumin ratio demonstrated in the patients with C3c in their sputum sol phase and also in patients with activated factor B supports the concept of complement induced inflammation with subsequent transudation of plasma proteins in these patients

This difference in albumin ratio could however not be demonstrated when the CF + P patients were compared with the CF-P patients The CF P patients number 18 and 22 (see Table 1) had C3c in their sputum sol phase and thus also had a relatively high albumin ratio The complement consumption observed in these patients could possibly be due to complement activation induced by complexes of IgG with protein A of *S aureus* as they had lung infection with these bacteria at the time of investigation (6 17)

Evidence for local production of the components of the alternative pathway has been presented by *Robertson et al* (13) This question was not investigated in our study – but it is remarkable that the 16 patients with factor B all had the activated form as well – while none of the remaining 11 patients had any signs of factor B

This might indicate that activation of factor B (and also of C3) takes place below the mucosal surface of the lungs thereby mediating the transudation of complement components and other serum components to the mucosal surface

Complement C3 and C4 are known as acute phase reactants No significant difference between C3 or C4 values could however be observed between the CF + P and the CF P patients probably because we are dealing with chronically infected patients These results are in accordance with our previous findings (15)

While no evidence of local pulmonary production of complement factors was found this study shows that complement mediated inflammatory reactions

play a role in the patogenesis of pulmonary tissue damage in patients with cystic fibrosis and it supports the concept of chronic *P aeruginosa* lung infection as an immune complex disease in CF patients

This work was supported by the *Danish Medical Research Council* (project no 512 7193 and 512 6623) the *National Danish Association against Cystic Fibrosis* and the *Poul Bergsøe Foundation*

We thank *Anni Bethien Ellinor Ward Petersen Aase Stricker* and *Astrid Viderø* for skilful technical assistance

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COMPLEMENT RECEPTORS IN HUMAN PERIPHERAL NERVE TISSUE

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Nyland H Matre R & Tonder O Complement receptors in human peripheral nerve tissue Acta path microbiol scand Sect. C 87 7–10 1979

Cryostat sections of human peripheral nerve tissue adsorbed sheep erythrocytes sensitized with antibody and human complement (EAC) Activated complement was essential for the reaction to occur The receptor had specificity for the C3b fragment. In order to obtain binding to nerve tissue the indicator cells had to be coated with more complement than that required for binding to C3b receptors in spleen and renal tissue The receptors are located within the nerve fascicles and are probably of glycoprotein nature The receptors for C3b in peripheral nerve tissue may be of significance in the deposition of immune complexes which may play a role in acute polyradiculoneuritis

Key words C receptors peripheral nerve tissue

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Receptors for the third component of complement (C3) have earlier been detected on human erythrocytes (12) granulocytes (7) monocytes and macrophages (8 9) and B lymphocytes (2) Recently complement receptors have also been detected in human renal glomeruli (6 11) and on human diploid fibroblasts in culture (16 17)

Data in this report indicate that receptors for activated complement are present in human peripheral nerve tissue

MATERIALS AND METHODS

Tissues

Peripheral and central nerve tissue were obtained from autopsies performed at the Department of Pathology 9–20 h after the death of patients who had cardiac infarction but no systemic disease Peripheral nerve tissue from rabbits rats and guinea pigs were obtained from laboratory animals Kidney and spleen were provided by the Department of Surgery Small pieces of the various samples were quickly frozen and mounted on specimen holders for the preparation of tissue sections Sections 6–8 µm thick were cut in a cryostat and placed on 22×40 mm coverglasses Tissue and cryostat sections were stored in sealed containers at −25° C For histological investigations tissues were fixed in 4 per cent neutral formaldehyde

Sera

Normal human sera obtained from blood donors were used as sources of complement (C) Complement was inactivated by heating the sera at 56° C for 30 min Heated human serum absorbed with sheep erythrocytes was used as C3b inactivator reagent (13)

Antisera to sheep erythrocytes were raised in rabbits and serum samples were collected at various intervals after immunization to obtain IgM and IgG antibodies

Unlabelled rabbit antisera to human immunoglobulins (polyvalent) C1q C3 C4 and albumin and fluorescein isothiocyanate (FITC) labelled antisera to human immunoglobulins and C3 were purchased from DAKO immunoglobulins a/s (Copenhagen Denmark)

Immunoglobulins

Human IgG (Fraction II 16 5 per cent solution) was purchased from AB Kabi (Stockholm) and human albumin from Behringwerke (Marburg Lahn West Germany) Aggregated IgG was prepared by heating the

solutions at 63° C for 15 min Rabbit IgM antibody to sheep erythrocytes was isolated from early immune response antisera by gel filtration on Sephadex G 200 equilibrated with phosphate buffered saline pH 7 2 (PBS)

Erythrocytes
Whole blood from sheep was collected in equal amounts of Alsever s solution and kept at 4° C Before use the erythrocytes were washed 3× in PBS with centrifugation at 800 × *g* for 5 min and final packing at 1 000 × g for 10 min

Indicator Cells
Sheep erythrocytes (E) were sensitized with rabbit antibodies (A) of IgM class (IgMEA) or IgG class (IgGEA) in varying amounts expressed as agglutinating units One agglutinating unit is defined as the amount of the highest dilution of A that agglutinates an equal amount of a 1 per cent suspension of E The mixture of E and A were incubated at room temp for 30 min The EA were washed twice and made up to a 2 per cent suspension in barbital (Veronal) buffered saline pH 7 2 containing 0 15 mM $CaCl_2$ 0 5 mM $MgCl_2$ and 0 01 per cent gelatin (GVB)

E sensitized with 1/4 agglutinating unit of IgM were used to prepare complement coated erythrocytes (EAC) Zymosan treated human sera which retain considerable C3 activity with low C5 activity (18) were used as a source for C Equal volumes of 2 per cent IgMEA and varying dilutions of C in GVB (see Results) were mixed and incubated for 10 min at 37° C Under these conditions the effect of C3b inactivator (KAF) is negligible The cells were washed twice and finally made up to a 1 per cent suspension in GVB (EAC3b) IgMEA incubated in inactivated zymosan treated human serum and EAC3b incubated in C3b inactivator reagent (EAC3d) served as controls (11)

The procedure with tissue sections was the same as described previously using the closed chamber technique (11 15) Briefly the concavity of a microculture slide was filled with a 1 per cent suspension of the various indicator cells A coverglass with a cryostat section was pressed into place on to the slide with the tissue section in the centre of the concavity The slide was placed with the coverglass facing downwards to allow the indicator cells to settle on the tissue section After 30 min at room temp the slides were turned over and left so that the indicator cells detached from the coverglass and from non reactive tissue settling into the concavity of the microculture slides The degree of haemadsorption (3 + 2 + 1 +) was recorded microscopically when the coverglass around the tissue was free of erythrocytes Absence of haemadsorption was designated as − Fixation and staining of the preparations were performed as described previously (9)

Pretreatment of Tissue Sections
Sections were incubated for 30 min at room temp with rabbit antisera to human immunoglobulin classes C1q C3 C4 and albumin or with varying concentrations of trypsin (once crystallized DCC treated (Sigma Chemical Co St Louis Mo USA)) or periodic acid in PBS The sections were washed in PBS and tested against the various indicator cells

Direct Immunofluorescence Test
Cryostat sections were incubated for 30 min in a moist chamber at room temp with FITC labelled rabbit antisera to human immunoglobulins (polyvalent) and to C3 diluted 1 in 2 in PBS The sections were then washed in PBS and mounted in 50 per cent glycerol in PBS and examined under a Leitz Orthoplane microscope equipped with a HBO−200 mercury lamp

Test for Inhibition of Haemadsorption
In these experiments the EAC3b were suspended in the preparations to be tested for inhibiting activity If the preparations contained agglutinins to E these were exhausted by absorption with E before use

RESULTS

Sections of human peripheral nerve tissue adsorbed EAC3b in a focal pattern (Fig 1a) Apparently the binding occurred within the nerve fascicles Sections from different nerves both somatic and autonomic as well as sections prepared from specimens at various levels of the nerves possessed this activity Specimens from 10 individuals whose ages ranged from newborn to 80 years all gave similar reactions No reaction occurred with sections from brain medulla and optic nerve The haemadsorption stopped abruptly at the transition from peripheral to central nerve tissue (Fig 1b)

A number of experiments were designed to study the factor(s) which mediated the haemadsorption Unsensitized E or E sensitized with as much as 2 agglutinating units of IgG or IgM IgMEA incubated in heated human serum did not adhere Presumably activated complement was necessary for the reaction to occur

That C3 was essential for the binding was indicated by the following results Antiserum to C3 agglutinated the EAC3b The EAC3b gave a positive immune adherence test and adhered to glomeruli in sections of human kidney (11) and to the C3b receptor positive areas in sections of human spleen (3 4) EAC3b treated with C3b inactivator reagent gave no reaction in the immune adherence test and did not adhere to sections of nerve tissue or of renal glomeruli However they were agglutinated by anti C3 and adhered to the C3d receptor positive follicular areas in sections of spleen (3 4) Assumably the binding of EAC3b to nerve tissue is mediated by the C3b component of complement indicating a C3b receptor in peripheral nerve tissue The results above indicated that the receptor is widely spaced within the nerve fasicles

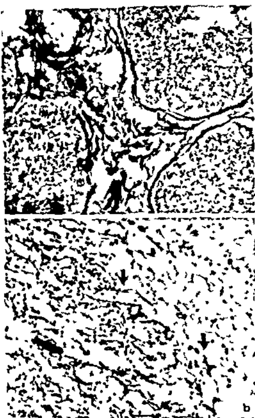


Fig 1 Adherence of IgMEAC (complement-coated sheep erythrocytes sensitized with IgM antibodies) to sections of nerve

it is to peripheral nerve tissue and not to the central nerve tissue (× 400)

The binding activity of this receptor was then compared to the binding activity of previously recognized receptors in glomeruli and spleen using EAC3b coated with varying amounts of C (*Table 1*) In order to obtain binding to nerve tissue the indicator cells had to be coated with more complement than that required for binding to spleen and renal tissue Consequently the C3b receptor in nerve tissue has less binding activity

The direct immunofluorescence technique did not reveal *in vivo* bound immunoglobulin or C3 in sections of normal nerve tissue Preincubation of sections with rabbit antiserum to human immunoglobulins, C1q, C3, C4, and albumin did not affect the binding of EAC3b The adherence of EAC3b was not inhibited by native or aggregated human IgG, even at a concentration of 16 g/l Tissue treated with trypsin at a concentration of 0 005 mg/ml and periodic acid at a concentration of 0 1 mM had no receptor activity Formaldehyde fixed and haematoxylin and eosin (H & E) stained sections showed normal structure of those peripheral nerves studied

EAC did not adhere to sections of peripheral nerve tissues from rabbits and guinea pigs

DISCUSSION

The results obtained showed that human peripheral nerve tissue obtained from individuals of various age possess receptors for activated human complement The receptors were found at various levels both in somatic and autonomic nerve tissue The receptors were localized diffusely within the nerve fascicles whereas the connective tissue compartments showed no receptor activity That the adsorption of EAC3b indicator cells stopped abruptly at the transition from peripheral to central nerve tissue, seems to indicate that the receptors are associated with the Schwann cells which ensheath the axons in the peripheral nervous system (1) The complement receptor described on fibroblasts cultured from

have the capacity to differentiate to connective tissue cells (1)

The results further strongly indicated that the receptors had specificity for C3b Only the complete

TABLE 1 *Adsorption of EAC3b to Sections of Peripheral Nerve Tissue Renal Tissue and Spleen The EAC3b were Prepared with Varying Amounts of Human Complement (C)*

Type of tissue	Dilutions of zymosan treated human serum 1 in								
	2	4	8	16	32	64	128	256	512
Peripheral nerve	2 +	2 +	1 +	1 +	1 +	–	–	–	–
Normal kidney	3 +	3 +	3 +	3 +	3 +	2 +	2 +	1 +	–
Normal spleen	3 +	3 +	3 +	3 +	3 +	2 +	2 +	1 +	–

EAC3b indicator system mediated the haemadsorption That C3b was avaible on the cells was evident from agglutination by antiserum to C3 immune adherence and adsorption to known C3b receptor areas in kidney and spleen C3d can not be involved since EAC3b treated with C3b inactivator gave no reaction That C3 was still on the indicator cells was evident from the agglutination obtained with the anti C3 serum and from the reactivity with the C3d receptor positive follicular areas of spleen (3 4)

The receptors were sensitive to trypsin and periodic acid at low concentrations The sensitivity to trypsin is consistent with the properties reported for complement receptors on monocytes (8) erythrocytes (12) and human renal glomeruli (11) The sensitivity to trypsin and periodic acid indicated that the receptor is a protein or a glycoprotein The results of experiments using EAC3b coated with varying amounts of complement indicate that the nerve C3b receptor has a lower binding activity than the C3b receptors in spleen and glomeruli

The receptor for C3b in peripheral nerve tissue may be of significance for the *in vivo* deposits of immune complexes which may play a role in for instance acute polyradiculoneuritis (5 10 14)

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INCIDENCE AND TITRES OF SMOOTH-MUSCLE ANTIBODIES IN HUMAN SERA

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Andersen P Incidence and titres of smooth muscle antibodies in human sera Acta path microbiol scand Sect C 87 11–16 1979

[illegible]

[illegible] gravis progressive muscular dystrophy atopic dermatitis and mycoplasma pneumoniae infection) the prevalence did not differ from that of controls (3 6%) IgM SMA were found more often in CAH (28 6%) [illegible]

[illegible]

Key words Smooth muscle antibodies incidence titres human sera

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Sera with high titres of antibodies to smooth muscle (SMA) are often derived from patients with chronic active hepatitis (CAH) (3 6 13 15 19) However SMA in low titres have also been found in some acute infections (5 12) and in other liver diseases (8 15) and may possibly be found also in patients with diseases which may be associated with liver disorders such as ulcerative colitis and Sjogren s syndrome (10 16) SMA in chronic liver diseases are predominantly of the IgG class and they have been shown to react with actin from both skeletal and smooth muscle (3) SMA might [illegible] om patients with disorders [illegible]

[illegible] determinations it is necessary to know the incidence and titre range of the antibodies in patients with different diseases and in normal controls In this study the incidence and titres of SMA of the IgG IgA and IgM class were determined in a variety of diseases and the results were compared with the findings in normal controls and in patients with CAH The aim of the investigation was to study whether the demonstration of SMA could be of diagnostic value

EAC3b indicator system mediated the haemadsorption That C3b was avaible on the cells was evident from agglutination by antiserum to C3 immune adherence and adsorption to known C3b receptor areas in kidney and spleen C3d can not be involved since EAC3b treated with C3b inactivator gave no reaction That C3 was still on the indicator cells was evident from the agglutination obtained with the anti C3 serum and from the reactivity with the C3d receptor positive follicular areas of spleen (3 4)

The receptors were sensitive to trypsin and periodic acid at low concentrations The sensitivity to trypsin is consistent with the properties reported for complement receptors on monocytes (8) erythrocytes (12) and human renal glomeruli (11) The sensitivity to trypsin and periodic acid indicated that the receptor is a protein or a glycoprotein The results of experiments using EAC3b coated with varying amounts of complement indicate that the nerve C3b receptor has a lower binding activity than the C3b receptors in spleen and glomeruli

The receptor for C3b in peripheral nerve tissue may be of significance for the *in vivo* deposits of immune complexes which may play a role in for instance acute polyradiculoneuritis (5 10 14)

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INCIDENCE AND TITRES OF SMOOTH-MUSCLE ANTIBODIES IN HUMAN SERA

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Andersen P Incidence and titres of smooth muscle antibodies in human sera Acta path microbiol scand Sect C 87 11–16 1979

nervous system (19%) In other diseases (Sjøgren s syndrome ulcerative colitis regional enteritis myasthenia gravis progressive muscular dystrophy atopic dermatitis and mycoplasma pneumoniae infection) the prevalence did not differ from that of controls (3 6%) IgM SMA were found more often in CAH (28 6%) in infections of the central nervous system (14 3%) and in mycoplasma pneumoniae infections (13%)
detected only in a
diagnosis of HBA.
diagnostic value in chronic hepatitis

Key words Smooth muscle antibodies incidence titres human sera

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Sera with high titres of antibodies to smooth muscle (SMA) are often derived from patients with chronic active hepatitis (CAH) (3 6 13 15 19)

patients with diseases which may be associated with liver disorders such as ulcerative colitis and Sjogren s syndrome (10 16) SMA in chronic liver diseases are predominantly of the IgG class and they have been shown to react with actin from both skeletal and smooth muscle (3) SMA might therefore also occur in sera from patients with

determinations it is necessary to know the incidence and titre range of the

variety of diseases and the results were compared with the findings in normal controls and in patients with CAH The aim of the investigation was to study whether the demonstration of SMA could be of diagnostic value

MATERIALS AND METHODS

Subjects

Sera from 182 patients with a variety of diseases were studied and the findings were compared with those of 582 control persons and 7 patients with CAH Some of the sera had been stored at −20° C for up to 6 years The subjects were divided into the following groups

Sjøgren s syndrome This group consisted of 15 female patients aged 41–81 years All patients fulfilled two of the following three criteria Keratoconjunctivitis sicca xerostomia and rheumatoid arthritis Keratoconjunctivitis sicca was based on a Shirmer s test and staining with Rose Bengal xerostomia was based on sialometry or histological examination of a biopsy from the lip (4)

Ulcerative colitis This group comprised 18 female and 11 male patients aged 16–63 years The diagnosis was based on history radiological findings proctoscopy and a biopsy from the rectal mucosa

Regional enteritis This group comprised 5 female and 3 male patients with an age range of 26–74 years The diagnosis was based on a history of intermittent chronic diarrhea fever weight loss and abdominal pain and on radiological findings Sera from patients with regional enteritis ulcerative colitis and CAH were kindly supplied by Dr *Karin Ladefoged* Medical Department P Randers City Hospital Denmark

Myasthenia gravis This group comprised 20 female and 11 male patients aged 18–72 years The diagnosis was based on a clinical examination and a positive edrophonium chloride (Tensilon) test and in some cases it was confirmed by typical electromyographic findings All patients attended the out patient clinic at the Neurological Department Aarhus Municipal Hospital Aarhus Denmark

Progressive muscular dystrophy This group consisted of 11 male patients aged 8–26 years The diagnosis was based on history and clinical examination Ten patients were from a nursing home and the oldest attended a neurological out patient clinic

Atopic dermatitis This group comprised 16 female and 10 male patients aged 9–64 years The diagnosis was based on clinical examination They were all admitted to the Department of Dermatology Marselisborg Hospital Aarhus Denmark

Syphilis This group consisted of 4 female and 13 male patients aged 23–60 years The diagnosis was based on demonstration of *Treponema pallidum* by dark field microscopy or a positive *Treponema pallidum* immobilization test

Infections of the Central Nervous System (CNS) This group comprised 21 patients Nine cases were diagnosed as acute lymphocytic meningitis six as acute encephalitis four as polyradiculitis one as transverse myelitis and one as cerebellitis The patients were examined at weekly intervals from one to four times during the course of disease and a total of 45 serum samples were investigated

Mycoplasma pneumoniae infection A total of 53 sera from 23 patients with mycoplasma pneumoniae infection were investigated The diagnosis was based on demonstration of a significant (4 fold) increase or decrease in cold agglutinin and/or mycoplasma antibody titre Mycoplasmas were isolated in 10 cases but could not be isolated in 7 cases In 6 cases no attempts were made to isolate mycoplasmas One to four serum samples obtained from each patient during the course of disease were studied Sera from these patients and from patients with infections of CNS were kindly supplied by Dr *Klaus Lind* Statens Seruminstitut Copenhagen

Chronic active hepatitis (CAH) This group consisted of 7 patients aged 22–71 years Three patients had Hepatitis B associated antigen (HBAg) in serum while 4 were HBAg negative A liver biopsy was performed in all patients and liver bioptic changes corresponding to chronic aggressive hepatitis as described by *De Groote et al* (7) were found in all cases They had all had signs of liver disease without recovery for at least 3 months

Controls This group comprised 275 females and 307 males aged 5 months – 76 years This material has been published elsewhere (2) and it consisted of blood donors laboratory technicians medical students and hospitalized patients without autoimmune infectious or liver diseases

Control sera Six sera containing IgG or IgM SMA were used as positive control sera These sera had been stored at −20° C for up to 32 months and they were titrated at several occasions during the study

Antibody Tests

Antibodies were demonstrated by indirect immunofluorescence (IIF) as previously described (1 2) Unfixed cryostat sections 4 µm thick of rat stomach and rat kidney were used as antigens Antibodies reacting with gastric smooth muscle (SMA) renal vessel walls and renal glomeruli (GA) were recorded Both polyspecific fluorescein isothiocyanate (FITC) conjugated antihuman immunoglogulin and monospecific conjugates against human IgG IgA and IgM (Wellcome Research Laboratories England and Behringswerke AG Germany) were employed for the antibody tests The monospecific conjugates were specific for heavy chains and they did not cross react in gelprecipitation The molar fluorescein protein ratios of the conjugates were between 1 3 and 4 6 and they were used in a dilution corresponding to an antibody content of 1 4 units/ml In experiments where polyspecific conjugates were employed three positive control sera containing antibodies of the IgG IgA and IgM class were included while only one positive controlserum was included in experiments where monospecific conjugates were used All sera were initially tested at a dilution of 1 10 Sera from patients with myasthenia gravis progressive muscular dystrophy syphilis and some of the controls were initially tested using a polyspecific conjugate while all other sera were tested using monospecific conjugates All sera which initially were positive or doubtfully positive were titrated in doubling dilutions starting with 1 10 and they were investigated for antibodies of the IgG IgA and IgM class by means of monospecific conjugates The optic system used was a Zeiss fluorescence microscope equipped with an HBO 200 mercury

exiter filter was an interference filter (400–500 nm) adjusted for fluorescein and the barrier filter was matched to the interference filter

RESULTS

Effect of Storage of Serum on SMA Titres

Several of the serum samples investigated in this study had been stored at −20° C for up to 6 years In order to study whether the storage of serum might alter the SMA titres three IgG SMA positive and three IgM SMA positive sera were titrated on several occasions It appears from Table 1 that no significant changes in titres could be demonstrated after storage of serum for up to 32 months This suggests that the antibody findings in sera which had been stored frozen for about 2 1/2 years may not differ from the results which would have been obtained if fresh serum samples had been used

Incidence and Titres of SMA

The frequencies and titres of SMA in patients and controls are shown in Table 2 IgG and IgM SMA occurred in both patients and controls and antibodies of both immunoglobulin classes were often found together IgA SMA *in a titre of 10* was demonstrated in only one [illegible] als [illegible]

patients with HBAg negative CAH but not in any other patients or controls The fact that the IgG SMA titres in the three HBAg positive CAH cases were below 40 is especially noteworthy IgG SMA titres of 80 were detected in one of the controls in one patient with syphilis and in one patient with myasthenia gravis Serum from the latter patient reacted with gastric smooth muscle in a titre of 80 while it did not stain vessel walls All other positive sera revealed similar titres with both antigens IgG

TABLE 1 *SMA Titres after Storage of Serum at −20° C*

Serum	Ig Class	Months of storage 0	1	4	9	13	20	24	32
x–44	IgG	80			40				80
x 126	IgG	160	320					160	
6177	IgG	40		40					
R–33	IgM	20		20		40		20	
R–34	IgM	40		20		20			
R–255	IgM	80	80				40	80	

SMA were detected in 6 of 7 patients with CAH (85 7%) A significantly increased frequency was also found in patients with infections of CNS (19%) and in patients with syphilis (16 7%) (Fischer s exact test p = 0 01 and p = 0 03, respectively) The incidence in other diseases did not differ from that of controls

IgM SMA titres above 20 were demonstrated in only two controls and in none of the patients However IgM SMA in low titres were found significantly more often in patients with infections of CNS (14 3%) in patients with mycoplasma [illegible]

IgM SMA also occurred more frequently in patients with syphilis than in controls but this difference was not significant The incidence in other diseases was of the same range as in controls

In 21 patients with infections of CNS who were studied during the course of illness SMA were *found in 5 (23 8%)* In these 5 patients SMA were present at the beginning of disease and disappeared from serum within a few weeks (Table 3) The titres were low and antibodies of both the IgG (4 patients) and IgM (3 patients) class were demonstrated One of the SMA positive patients had acute encephalitis and infectious mononucleosis another had acute encephalitis complicating measles while the etiology of the 3 remaining SMA positive cases was undetermined In 23 patients studied during the course of a mycoplasma pneumoniae infection SMA were found in 13% but it was not possible to show that the antibodies had developed during the course of disease

Incidence of Glomerular Antibodies

Glomerular antibodies were demonstrated in 4 of 182 patients (2 2%) with various diseases and in 14 (2 4%) of the controls (2) In 3 of the patients glomerular antibodies occurred together with SMA and both types of antibodies were of the same immunoglobulin class The fourth patient who was SMA negative had glomerular antibodies in a titre of 40 Nine of the controls with glomerular antibodies also had SMA while IgG glomerular antibodies in titres of 10–80 were detected in five SMA negative controls In four HBAg negative patients glomerular antibodies were detected in titres of 40–320 which were one or two serum dilutions lower than the SMA titres In the HBAg positive CAH cases glomerular antibodies in a titre of 10 were found in only one patient with SMA in a titre of 20

TABLE 2 *Incidence and Distribution of Titres of SMA in Various Diseases*

Diagnosis	No tested	No with SMA	%	No with IgG-SMA	%	IgG-SMA titres 10	20	40	80	160	320	640	1280	No with IgM SMA	%	IgM-SMA titres 10	20	40	80
Sjøgren s syndrome	15	2	13 3	2	13 3	1	1							1	6 7		1		
Ulcerative colitis	29	2	6 9	1	3 4	1								1	3 4		1		
Regional enteritis	8	0	0	0	0									0	0				
Myasthenia *gravis*	31	2	6 5	2	6 5		1		1					0	0				
Progressive muscular dystrophy	11	0	0	0	0									0	0				
Atopic dermatitis	26	2	7 7	1	3 8	1								1	3 8		1		
Syphilis	18	4	22 2	3	16 7		1	1	1					2	11 1	2			
Infections of CNS	21	5	23 8	4	19 0	3		1						3	14 3	3			
Mycoplasma pneumoniae infection	23	3	13 0	2	8 7	1		1						3	13 0	2	1		
Chron act hepatitis	7	6	85 7	6	85 7	1	1			2	1		1	2	28 6	1	1		
Controls	582	32	5 5	21	3 6	14	6		1					15	2 6	7	6	2	

TABLE 3 *SMA Titres in Five Patients with Infections of the Central Nervous System*

Patient	Antibody	First sample	Weeks after first sample 1	3–4	7	Diagnosis
A	IgG SMA	10	10	–		Polyradiculitis
	IgM SMA	10	–	–		
B	IgG SMA	–	10	–		Acute lymphocytic meningitis
	IgM SMA	–	–	–		
C	IgG SMA	40	10	–	–	Acute encephalomyelitis Measles
	IgM SMA	10	–	–	–	
D	IgG SMA	10	–		–	Acute encephalitis
	IgM SMA	–	–		–	
E	IgG SMA	–	–	–		Acute encephalitis *Infectious mononucleosis*
	IgM SMA	10	10	–		

– titre below 10

DISCUSSION

The finding of a myasthenia gravis serum which reacted with rat stomach muscle but not with rat renal vessel walls supports the assumption that SMA with different specificities occur (3 6) *Sera* with SMA in high titres often react with renal glomeruli and *absorption experiments* with sera from CAH patients have shown that the antibodies react with actin *present in both* gastric *smooth* muscle and glomeruli (3) However the finding of *glomerular antibodies in* SMA *negative* sera indicates that some antibodies to glomeruli may react with *antigens other than actin*

SMA in infectious diseases are often of the IgM class *but IgG antibodies* also occur They are present in the early phase of illness and usually they disappear *from serum* within a few months (5 11 12) This was also the case in patients with infections of CNS in the present investigation One of the SMA-positive patients in this group had measles and another had infectious mononucleosis and both of these diseases have previously been found associated with SMA (11 12) However SMA have also been found in other infectious diseases *Biberfeld & Sterner* (5) thus found an increased incidence of IgM SMA in mycoplasma *pneumoniae infection* The antibody titres rose at the beginning of disease and decreased later during *the course of illness* An increased incidence of IgM SMA in mycoplasma pneumoniae infection was also found in the present study but it was not possible *to demonstrate that the antibodies had* developed during the course of infection

An increased incidence of IgG-SMA but not of IgM SMA was demonstrated in syphilis but the titres did not exceed those found in controls The reason for the developm... of SMA ...

... 19 % of patients with Sjøgren s syndrome and this was significantly higher than i controls SMA also occurred with increased frequency in this study but the difference was not significant This could be due to the small number of patients studied to differences in the sens t v ty of ...

...syndrome rheumatoid arthritis *and sicca syn*drome *Webb et al* (18) thus found a greater prevalence of abnormalities *in liver function* tests in those with SMA than in those without suggesting that liver disorders *might be responsible* for the development of SMA However *Perrett et al* (16) could not demonstrate *any correlation* between abnormal liver f... colitis ... of SM... functior ...

TABLE 2 *Incidence and Distribution of Titres of SMA in Various Diseases*

Diagnosis	No tested	No with SMA	%	No with IgG-SMA	%	IgG-SMA titres 10	20	40	80	160	320	640	1280	No with IgM-SMA	%	IgM-SMA titres 10	20	40	80
Sjøgren s syndrome	15	2	13 3	2	13 3	1	1							1	6 7		1		
Ulcerative colitis	29	2	6 9	1	3 4	1								1	3 4		1		
Regional enteritis	8	0	0	0	0									0	0				
Myasthenia gravis	31	2	6 5	2	6 5		1		1					0	0				
Progressive muscular dystrophy	11	0	0	0	0									0	0				
Atopic dermatitis	26	2	7 7	1	3 8	1								1	3 8		1		
Syphilis	18	4	22 2	3	16 7		1	1	1					2	11 1	2			
Infections of CNS	21	5	23 8	4	19 0	3		1						3	14 3	3			
Mycoplasma pneumoniae infection	23	3	13 0	2	8 7	1		1						3	13 0	2	1		
Chron act hepatitis	7	6	85 7	6	85 7	1	1			2	1		1	2	28 6	1	1		
Controls	582	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]								15	2 6	7	6	2	

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MODULATION BY MACROPHAGES OF LYMPHOCYTE RESPONSES TO PHYTOHAEMAGGLUTININ, CONCANAVALIN A AND SEMIALLOGENEIC CELLS

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Hem E Modulation by macrophages of lymphocyte responses to phytohaemagglutinin concanavalin A and semiallogeneic cells Acta path microbiol scand Sect C 87 17–21 1979

To investigate the effect of macrophages on rat lymphocyte activation by phytohaemagglutinin (PHA) concanavalin A (Con A) and semiallogeneic cells (unidirectional MLR) increasing concentrations of peritoneal cells (PC) were added to cultures of lymph node lymphocytes Lymph node cell preparations contained 2–4% esterase positive cells (macrophages/monocytes) Any additional PC seemed only to inhibit MLR whereas optimal responses of PHA and Con A stimulated cultures occurred when 1–2% abd 3–7% PC respectively were added Ten percent PC markedly inhibited the response of both PHA and Con A stimulated cultures when added for the last 24 h of incubation Inhibitory cells were shown to belong to the glass wool adherent population of PC

Key words Lymphocyte activation macrophages

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Macrophages appear to play a regulatory role in lymphoproliferative responses induced by a variety of stimulating agents *in vitro* They enhance responses to antigens (13 17 19 21) allogeneic cells (1 4) and non-specific mitogens (6 18) and improve viability of lymphoid cells in culture (3) On the other hand several recent reports indicate that macrophages present in spleens and peritoneal washings of mice and rats can also inhibit lymphoproliferation (2 5 11 15 16 23 27–29) These dual effects of macrophages have been thoroughly reviewed by *Nelson* (14) Evidence is now emerging which indicates that enhancement will occur at lower concentrations and inhibition at higher concentrations of macrophages (6 11 18 24) The aim of the present investigation was to study the relation between the enhancing and the inhibitory effect of macrophages in relation to different types of lymphocyte stimulation and to find optimal macrophage/lymphocyte ratios for enhancement or inhibition of lymphocyte response The findings suggest that optimal macrophage/lymphocyte ratios vary according to the stimulant used

MATERIALS AND METHODS

Animals

Inbred locally grown [illegible]

[illegible] weeks were used

Cell Suspension

Lymph node cell suspension were prepared from mesenterial cervical and axillar nodes as previously described (7) Peritoneal cells (PC) were collected by washing the peritoneal cavity with 20 ml phosphate buffered saline (8) Neutral esterase staining (20) showed that lymph node cell preparations contained 2–4% positive cells (macrophages/monocytes) and in the PC suspensions 65–80% were heavily stained

The reason for development of SMA in infections is not known, but also in these diseases it might be linked with liver cell damage (12) However, in mycoplasma pneumoniae infection no correlation could be established between the occurrence of SMA and elevated transaminase values (5) This further suggests that the occurrence of SMA is not necessarily related to liver involvement

SMA were not detected in progressive muscular dystrophy and they were found with equal frequencies in myasthenia gravis and controls (19) Thus the development of SMA could neither be related to disorder of the muscular system

The same immunofluorescence technique used in this investigation was also employed to detect SMA in sera from patients with various liver diseases, including CAH, primary biliary cirrhosis and alcoholic liver disease (14) IgG SMA titres above 80 occurred in only HBAg negative CAH, and the titres decreased in patients treated with immunosuppressive drugs (17) These data together with the present findings support the assumption that sera with high IgG SMA titres are derived from patients with untreated HBAg-negative CAH

In infectious diseases IgM SMA titres very seldom exceed 80 (5 11, 12 15) and IgM SMA titres above 40 were not observed in this investigation Thus high SMA titres found with polyspecific conjugates are most probably due to IgG antibodies These titres would therefore be of diagnostic value equal to high IgG SMA titres

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TABLE 2 *Effect of Non adherent Peritoneal Cells (PC) Added to Lymph Node Cell Cultures Stimulated with PHA of Con A*

Exp no	Mitogen	^{3}H thymidine incorporation (cpm) ± 1 SD	
		Control	10% non adherent PC
1	PHA	77 604 ± 8 099	97,740 ± 7 856
	Con A	89 224 ± 6 992	128 572 ± 10 996
2	PHA	80 379 ± 4 743	93 158 ± 8 837
	Con A	96 421 ± 7 573	151 419 ± 11 367
3	PHA	60 901 ± 4 355	72 603 ± 6 560
	Con A	63 798 ± 4 879	93 009 ± 8 508

2×10^5 lymph node cells were cultured with mitogen for 48 h 2×10^4 non adherent PC were added at the initiation of the cultures Control cultures received only medium

experiments were performed with column purified lymph node cells (containing < 0 5% esterase positive cells) and the results obtained demonstrated the same trends as shown in Fig 1

2 *Kinetics of PHA and Con A-stimulated Cultures with and without PC*

Cultures with and without 10% PC were harvested at 24 48 72 and 96 h As seen in Fig 2 PHA stimulated PC-containing cultures showed only a weak response at all intervals The other stimulated cultures demonstrated the usual peak ^{3}H thymidine incorporation on days 2 and 3 On these days the isotope uptake by Con A was enhanced by addition of 10% PC PC thus apparently influence solely the magnitude of the response and not the kinetics

3 *Effect of PC when Added only for the last 24 h on Cultures Stimulated by PHA and Con A*

Ten per cent PC were added to stimulated cultures 24 h before harvest As shown in Table 1 PC then inhibited ^{3}H thymidine incorporation of both PHA and Con A-stimulated cultures

4 *Effect of Non adherent PC*

Ten per cent non adhere[illegible]

[illegible] incorporation by both PHA and Con A stimulated cultures (Table 2)

DISCUSSION

Stimulation of lymphocytes by thymus-dependent antigens and allogeneic cells has been demonstrated to be macrophage-dependent (1, 19, 21) Whether macrophages are necessary for activation by T-cell mitogens has remained controversial (22, 25) However, recent data presented by *Rosenstreich et al* (18) have shown that T-cell stimulation by mitogens is macrophage-dependent, but the concentration of macrophages necessary for mitogen stimulation may be lower than those required for stimulation by antigens and allogeneic cells (12, 19, 25) While these investigations focused on the minimal number of macrophages necessary to obtain activation, the present study was aimed at the determination of the number of macrophages required for optimal responses in these assays The results clearly showed that the requirement for macrophages varied according to the mitogen used Thus optimal responses after stimulation by PHA and Con A occurred when 1–2% PC and 3–7% PC respectively were added The peritoneal cells added seemed to influence only the magnitude of the response and not the time course of maximal stimulation by the mitogens Seemingly in contrast to these findings MLR were only inhibited by additional peritoneal cells Lymph node cell preparations contained 2–4% esterase positive cells (macrophages/monocytes) and this is propably enough for optimal stimulation Any excess macrophages will then inhibit proliferation Optimal concentrations of peritoneal cells enhanced lymphocyte activation by Con A more than by PHA, and ^{3}H thymidine uptake in these Con A-stimulated cultures was much higher than that of corresponding PHA-stimulated cultures (Fig 1) These findings may be related to the finding in mice of two T-cell mitogen responsive populations one that is activated by PHA [illegible]

[illegible] observations have formerly been made in rats by *Keller* (11) He reported that 1–4% »activated« peritoneal macrophages enhanced the response to Con A, whereas 10% or more were clearly inhibitory Inhibition by normal macrophages was demonstrated at the highest macrophage doses only This is apparently in contrast to the present findings However as he used plated macrophages in monolayers and spleen cells which contain higher concentrations of macrophages than lymph node cells, the results are not quite comparable Inhibitory effects on lymphoproliferation have also been

Cell Cultures

Cell cultures were performed as previously described (7) Briefly 150 µl cultures of 2 × 10^5 cells in medium RPMI 1640 with bicarbonate buffer penicillin 100 U/ml streptomycin 100 µg/ml and 5% fresh rat serum (FRS) were maintained in round bottom microtitre plates Con A (Sigma Chemical Company St Louis Miss) and PHA (Phytohaemagglutinin P Difco Detroit Michigan) were added at doses giving maximal activation of lymph node cell cultures 6 25 µg/ml Con A and 0 3% of the reconstituted PHA solution In MRL 1 × 10^5 Hooded cells were mixed with an equal number of F_1 cells giving a one way reaction Mitogenstimulated cultures were incubated for 48 h (or as shown in Fig 2) and MRL for 96 h The cultures were pulsed with 1 25 µCi ^{3}H thymidine (specific activity 5 Ci/mmol The Radiochemical Centre Amersham England) for the last 4 h and harvested on a Multiple Cell Culture Harvester (Skatron A/S Lierbyen Norway) and the amount of ^{3}H thymidine measured in a scintillation counter

Removal of Adherent Cells

Cell suspensions were purified by incubation on glass wool columns for 45 min and non adherent cells were eluted (9)

RESULTS

1 The Effect of PC on ^{3}H thymidine Incorporation by Lymphocytes Stimulated with PHA Con A or Semi allogeneic Cells

The effect of PC on isotope uptake by stimulated lymph node cells is shown in Fig 1 Low numbers

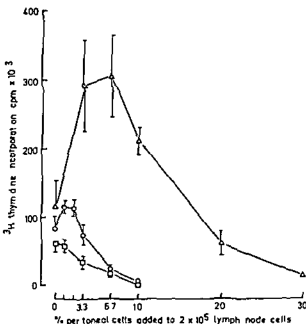


Fig 1 The effect of peritoneal cells (PC) on ^{3}H thymidine incorporation (cpm) of cultures stimulated by PHA (– O –) Con A (– △ –) and semi allogeneic cells (– □ –) The figures are mean values and standard deviations of triplicate (PHA Con A) or sixduplicate (MLR) cultures The cultures were incubated for 48 h (PHA and Con A) and 96 h (MLR)

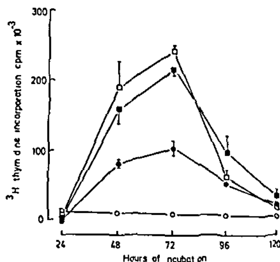


Fig 2 Kinetics of ^{3}H thymidine incorporation (cmp) by PHA stimulated cultures with (– O –) or without (– ● –) 10% PC and Con A stimulated cultures with (– □ –) or without (– ■ –) 10% PC The figures are mean values and standard deviations of triplicate cultures

of PC (< 3%) significantly enhanced ^{3}H thymidine incorporation of PHA stimulated cultures Higher concentrations were clearly inhibitory and at concentrations above 10% the response was very low In cultures stimulated with Con A maximal ^{3}H thymidine incorporation occurred when 3–7% PC were added and the isotope uptake was then more than doubled compared to control cultures without PC The ^{3}H thymidine incorporation by Con A stimulated cultures at optimal numbers of PC was twice that of corresponding optimal PHA stimulated cultures At concentrations above 30% PC also markedly inhibited the response of Con A

TABLE 1 *Effect of Peritoneal Cells (PC) Added to Lymph Node Cell Cultures Stimulated with PHA or Con A*

Exp no	Mitogen	^{3}H thymidine incorporation (cpm) ± 1 SD: Control	^{3}H thymidine incorporation (cpm) ± 1 SD: 10% PC for 24 h
1	PHA	82 603 ± 5 560	30 300 ± 10 356
	Con A	103 009 ± 9 508	47 508 ± 5 243
2	PHA	76 029 ± 11 334	44 917 ± 10 916
	Con A	89 772 ± 8 910	59 087 ± 13 349
3	PHA	73 798 ± 5 479	31 421 ± 5 054
	Con A	75 467 ± 9 218	32 634 ± 2 695

2 × 10^5 lymph node cells were cultured with mitogen for 48 h For the last 24 h 2 × 10^4 PC were added to the cultures Control cultures received only medium

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demonstrated with human peripheral blood macrophages (23)

It is also interesting to note that 10% peritoneal cells added for the last 24 h only, decreased ^{3}H-thymidine uptake by both PHA and Con A-stimulated cultures (Table 1) This finding, together with the demonstration of an optimal concentration of macrophages in the assays probably implies that the mechanism by which macrophages enhance stimulation by mitogens and allogenic cells is different from those inhibiting lymphoproliferation

The experiments shown in Table 2, provide evidence that at least the inhibitory effect by PC is caused by glass wool adherent esterase positive cells, probably macrophages The stimulatory effect in these experiments might be attributed to a small remainder of peritoneal macrophages and an increase in the absolute number of lymphocytes

In any case our studies have not only shown that the macrophage concentration is a parameter to be taken into account when interpreting lymphoproliferative response in general but also that macrophages at certain concentrations selectively may block some responses while only marginally affect others

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PRECIPITATING ANTIBODIES IN DERMATOPHYTOSIS DEMONSTRATED BY CROSSED IMMUNOELECTROPHORESIS

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Svejgaard E & Christiansen A H Precipitating antibodies in dermatophytosis demonstrated by crossed immunoelectrophoresis Acta path microbiol scand Sect C 87 23–27 1979

A total of 289 serum specimens from 262 patients with dermatophytosis were studied using crossed immunoelectrophoresis with an intermediate gel Rabbit antidermatophyte antisera were used as reference and the antigens were water soluble extracts of *Trichophyton* (T) *rubrum* *T mentagrophytes* *Microsporum* (M) *canis* or *Epidermophyton* (E) *floccosum* Each serum was tested against the dermatophyte antigen corresponding to the infectious agent of the patient in question Antidermatophyte antibodies could be demonstrated in 25 (9 5%) of the patients and these were particularly frequent in patients with highly inflammatory lesions and those with chronic dermatophytosis caused by *T rubrum* Antibodies were detected in all four patients with kerion Celsi. Nine patients five with acute dermatophytosis and four with chronic disease who had precipitating antibodies at the initial examination were studied several times during the course of the disease While the antibodies disappeared in all the patients with acute disease they persisted in those with chronic dermatophytosis The human antibodies showed marked cross reactivity with *T rubrum* *T mentagrophytes* *M canis* and *E floccosum*

Key words Dermatophytosis precipitating antibodies crossed immunoelectrophoresis

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Circulating antibodies have often been found in laboratory animals and humans with dermatophytosis There has been some disagreement however concerning the specificity and frequency of these antibodies This may be due to variations in a number of factors including 1) the immunological methods employed 2) the antigens used 3) the time during the course of infection when blood was drawn 4) the clinical manifestation whether chronic or acute superficial or deep 5) the immunological responsiveness of the infected person i e hyperreactivity as shown by the development of dermatophytids or hyporeactivity as seen in *immunodeficiencies* and 6) the dermatophyte species involved anthropophilic or zoophilic (4 6 7 8)

In the present study crossed immunoelectrophoresis (CIE) was used to determine the occurrence of precipitating antibodies in patients infected with *T rubrum* *T mentagrophytes* *T verrucosum* *T violaceum* *M canis* or *E floccosum* Standardized extracts of four of these species were used as antigens We also attempted to determine whether the presence of precipitating antibodies was related to infection with specific dermatophytes Finally we looked for an association between clinical manifestations of dermatophytosis and the production of precipitating antibodies

PATIENTS AND METHODS

Patients The study comprised 262 consecutive out patients with dermatophytosis caused by *T rubrum* (n = 132) *T mentagrophytes var interdigitale et var granulare* (n = 57) *T violaceum* (n = 1) *T verrucosum*

controls serum plus 3 6 ml 1% agarose The reference gel was made of 0 5 ml reference serum plus 7 ml 1% agarose (Fig 1)

A *positive* result was defined as the appearance of precipitates or reminiscences of precipitates (»inward feet«) in the intermediate gel A control plate with no serum in the intermediate gel was run in each experiment in order to avoid difficulty in the interpretation of precipitates in the upper part of the intermediate gel Precipitates in this area may originate in the reference gel

A high lipid content in the test serum is another possible source of error This may cause intense background staining thus obscuring weak precipitates (2)

RESULTS

Consecutive sera In 25 out of 262 sera as many as four precipitates were observed while no precipitates were seen in the remainder The results are listed and compared with the clinical manifestations of the infection in Table 1 Precipitating antibodies were rare in patients with uncomplicated superficial tinea (2 8%) while they were seen to develop in patients with acute inflammatory tinea pedis (62 5%) Precipitating antibodies were seen in the serum of all four children with deep dermatophytosis of the scalp *Humoral antibodies were seen twice as often* in patients with chronic localized *T rubrum* infections as in those with acute dermatophytosis *caused by T rubrum Several precipitates developed* in the serum of two patients with generalized dermatophytosis No precipitates were seen in sera from the controls

Precipitates Generally one specific precipitate was recognizable on each of the positive plates This precipitate appeared to be the same when the positive plates from patients were compared with control plates In sera from patients with chronic generalized dermatophytosis it was seen together with various other precipitates The precipitate is shown in Fig 1 with the arrow to the right

Follow up studies The CIE was repeated several times during the treatment of four patients with chronic dermatophytosis caused by *T rubrum* three children with kerion Celsi and two patients with acute inflammatory tinea pedis complicated by dermatophytids on the hands The results from three patients from this follow up study are shown in Table 2 Precipitating antibodies were consistently present in patients with *chronic infections* Among patients with acute infections these antibodies were demonstrated only in those with severe inflammation In all patients with *acute disease the* antibodies disappeared after three months



Fig 1 a and b Precipitating antibodies to *T rubrum* in a patient with generalized *T rubrum* infection studied by crossed immunoelectrophoresis with intermediate gel
a test plate
b control plate
Antigen Water soluble *T rubrum* antigens
Antibodies Intermediate gel on test plate Immunoglobulin fraction from patient. Intermediate gel on control plate *No serum*
Reference gel Immunoglobulin fraction from immunized rabbits
Precipitates and *»inward feet«* are shown by arrows

(n = 5), *E floccosum* (n = 53), or *M canis* (n = 14) Seventy of the patients with *T rubrum* or *T violaceum* had a chronic infection Eight patients with tinea pedis caused by *T mentagrophytes var granulare* also had vesicular dermatophytids on the hands Four children had kerion Celsi caused by the zoophilic dermatophyte species (*M canis, T verrucosum*, and *T mentagrophytes var granulare*) The remainder of the patients (n = 180) suffered from uncomplicated acute dermatophytosis with slight to moderate symptoms on glabrous skin, the groin and/or the feet

Controls Twenty females of the staff without signs of or history of dermatophytosis were included as controls

Antigens Water-soluble purified extracts of *T rubrum T mentagrophytes, M canis* and *E floccosum* were prepared as described in detail elsewhere (5) Briefly, lyophilized mycelia were disintegrated in a ball mill, and the extracts were adjusted with sterile water to contain 10 mg protein per ml The antigen solution contained from 25 to 35 different proteins depending on the species of the dermatophyte (5) As a rule the antigen used was extracted from the corresponding dermatophyte (Tables 1 and 2) In the follow-up study, other dermatophyte extracts were also used to investigate the possibility of cross reactivity

Sera i) Blood samples were drawn from the patients as soon as the diagnosis dermatophytosis was established, *viz* when the disease was active Samples were taken from a few of the patients at weekly or monthly intervals One sample was taken from each of the controls

ii) Reference serum pools were taken from rabbits immunized with extracts from the four dermatophyte species The immunoglobulin fractions from both the patient and control serum, as well as the rabbit serum were concentrated by salting out with $(NH_4)_2SO_4$ as described previously (5) This procedure increases the immunoglobulin concentration four to five times

Crossed immunoelectrophoresis with intermediate gel This technique differs from the conventional crossed immunoelectrophoresis in the interposition of an intermediate gel containing test serum between the gel containing the antigens separated in the first dimensional run and the gel containing reference serum The immunoelectrophoresis was carried out in 1 per cent agarose gel in barbital buffer Details regarding equipment and technique are described in *Axelsen et al* 1973 (2) and *Christiansen & Svejgaard* 1976 (5) A type of agarose with a low electroendosmosis (Litex, type HSA, Glostrup, Denmark) was used to counteract the tendency of immunoglobulins to move toward the cathode This can cause contamination of the intermediate gel with precipitates from the reference gel, thus increasing the difficulty of interpretation in this complex system of antigens In this study, the intermediate gel (dimensions 10 × 2 4 cm) consisted of 0 4 ml of the patients or

TABLE 1 *Circulating Antibodies against Dermatophytes in 262 Sera from Patients with Dermatophytosis*

Clinical manifestations		Dermatophyte	Total number of sera	Positive sera number	Positive sera per cent		Antigen
Acute, uncomplicated Tinea corporis, pedis and cruris		*T rubrum*	63	4	6 4	2 8	*T rubrum*
		T mentagrophytes var granulare et var interdigitale	48	0	0		*T mentagrophytes var granulare*
		E floccosum	53	1	1 9		*E floccosum*
		M canis	13	0	0		*M canis*
Acute Tinea pedis with dermatophytids on the hands		*T mentagrophytes var granulare*	8	5		62 5	*T mentagrophytes var granulare*
Kerion Celsi of the scalp		*T mentagrophytes var granulare*	1	1			*T mentagrophytes var granulare*
		T verrucosum	2	2			–
		M canis	1	1			*M canis*
Chronic infections	localized	*T rubrum*	67	9	13 4	15 7	*T rubrum*
		T violaceum	1	0	0		
	generalized	*T rubrum*	2	2	100		
Total			262	25		9 5	

those characteristic of acute or sub-acute contact dermatitis Immunofluorescence studies have shown immunoglobulins deposited in kerion Celsi lesions (9 11) Specific humoral antibodies as demonstrated by CIE may play a role in the elimination of fungal antigen in this type of infection The situation could be compared to the Arthus type reaction in which antibody excess leads to the precipitation of antigen antibody complexes at the site of infection

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TABLE 2 *Follow-up Studies of Circulating Antibodies against Dermatophytes in Three Patients with Dermatophytosis*

Patient	Clinical manifestation	Dermatophyte	Date of bleeding	Results	Antigen
KR	Acute Tinea pedis with dermatophytids	*T mentagrophytes var granulare*	19741127	1 precipitate	*T mentagrophytes*
				1 -	*T rubrum*
			19750218	– -	*T mentagrophytes*
			19750312	– -	*T mentagrophytes*
			19750710	– -	*T mentagrophytes*
HSL	Kerion Celsi	*T verrucosum*	19740729	1 precipitate	*T mentagrophytes*
				1 -	*T rubrum*
			19740823	1 -	*T mentagrophytes*
			19741017	1 -	*T mentagrophytes*
			19750123	– -	*T mentagrophytes*
BL	Chronic generalized	*T rubrum*	19721026	4 precipitates	*T rubrum*
				3 -	*T mentagrophytes*
				2 -	*E floccosum*
				1 -	*M canis*
			19760203	2 -	*T rubrum*
			19760611	2 -	*T rubrum*
			19760917	2 -	*T rubrum*

DISCUSSION

The CIE was used in this study of dermatophyte antigens because this method has been used successfully in the investigation of other microbial antigens such as those of *Candida albicans* (1, 2) This previous success was due mainly to the sensitivity of the method, making it possible to demonstrate a multitude of antigens in microbial extracts The use of CIE with an intermediate gel containing human sera has confirmed the sensitivity of the method in determining human immunological response This method is particularly recommended for studies of humoral antibody response to infections characterized by a complexity of antigens and low titre antibodies (2)

In contrast to what has been found previously in patients with candidiasis (1), the present study demonstrates that only a few of the previously detected 25–35 dermatophyte antigens are responsible for the precipitin production in human sera (Fig 1) The largest number of precipitates (four) was found in each of two patients with chronic generalized *T rubrum* infections (Table 2) Only patients with chronic infections had more than one precipitate, while sera from patients with acute dermatophytosis contained only one precipitate or »inward feet« The location of this precipitate was consistent from patient to patient

Cross reactivity between different Trichophyton species was demonstrated in the follow-up studies (Table 2) Extracts from *T rubrum* and *T mentagrophytes* could be used equally well to show humoral antibodies in patients infected with these two dermatophytes as well as *T verrucosum* Extracts with *E floccosum* and *M canis* also showed crossreactivity, though fewer precipitates were seen

Our results confirm the hypothesis that humoral antibodies are produced in patients with dermatophytosis The follow-up studies showed that these antibodies persist for a limited period after cure of the infections, while they persist indefinitely in patients with chronic dermatophytosis

The biological significance of humoral antibody production in dermatophytosis is still obscure It is generally assumed that cell-mediated immunity represents the major type of defence against dermatophytes (10), but it cannot be excluded that humoral antibodies also play a role Precipitins possibly in combination with non-specific serum factors, might inhibit the invasion of dermatophytes to deeper tissues (3) In patients with chronic dermatophytosis, the presence of fungal antigens in the skin might trigger several immunological mechanisms, i e both the cell mediated and the humoral antibody response

The mechanisms involved in the formation of kerion Celsi are not clear Mycological examination reveals hyphae and spores in the hairs, but these fungal elements are frequently difficult to demonstrate The histological manifestations are similar to

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ANTIBODY RESPONSES IN URINE AND SERUM AGAINST *ESCHERICHIA COLI* O ANTIGEN IN CHILDHOOD URINARY TRACT INFECTION

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Sohl Åkerlund A Ahlstedt, S Hanson L. Å & Jodal U Antibody response in urine and serum against *Escherichia coli* O antigen in childhood urinary tract infection Acta path microbiol scand Sect C 87 29–36 1979

Urine and serum antibodies against the infecting *E coli* strain were recorded using the enzyme linked immunosorbent assay in 22 infants and children with acute pyelonephritis or cystitis In most patients with pyelonephritis pronounced increases in the urine as well as the serum antibody levels were recorded in contrast to the patients with cystitis High IgG IgA and secretory IgA antibody levels were detected in the urine samples from 11 of 13 girls with pyelonephritis Occasionally IgM antibodies were also demonstrated in the urine from some of these patients Increased serum IgG IgA and IgM antibody levels were noted in the patients with pyelonephritis and secretory IgA antibodies could also be detected in the serum from most of these patients Comparison of the antibody levels in urine and serum revealed that secretory IgA antibodies appeared earlier in the urine than in the serum from 5 of 15 patients with acute pyelonephritis suggesting a local antibody production within the urinary tract The peak of the urine IgA antibody levels was reached within 10 days after onset of infection in 8 of 13 girls with pyelonephritis The patients with acute cystitis had low urine and serum antibody levels throughout the course of infection Five of 7 patients with cystitis had urine SIgA antibodies in the absence of such serum antibodies indicating a local antibody formation in the lower urinary tract of these patients

Key words Antibody responses *Escherichia coli* O antigen childhood urinary tract infection

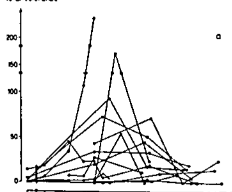
A Sohl Åkerlund Department of Immunology Institute of Medical Microbiology University of Goteborg Guldhedsgatan 10 S–413 46 Goteborg Sweden

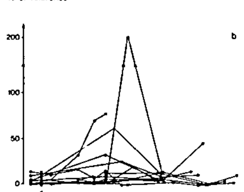
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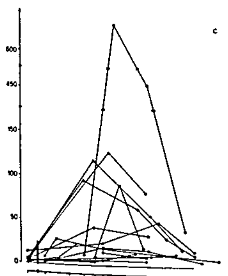
In most studies of the immune response during urinary tract infections (UTI) the serum antibodies have been investigated Such antibodies can be useful for the level diagnosis of UTI (11 27) and have also been found protective in experimental hematogenous infections in rabbits (18) Since UTI in humans is believed to be mainly of the ascending type antibodies detectable in the urine must also be considered Urine antibodies against the infecting organism have been reported to occur in patients with acute pyelonephritis (8 12 28) as well as in patients with acute cystitis (5) and asymptomatic bacteriuria (12) The antibodies present in the urine may bind to the infecting bacteria and can be detected in the urine sediment from patients with UTI (15 25) Furthermore specific antibodies against *E coli* antigens have been reported to be synthesized in the urinary tract (4 19 26) Such urinary antibodies of the IgG and IgA classes were also shown by Smith and associates to be locally produced in the kidneys of rabbits with experimental pyelonephritis (22 23)

Most previous studies investigating antibodies in human urine have been performed on concentrated

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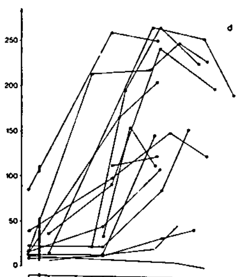






Fig 1 IgA and IgG antibody levels against the O antigen of the infecting *E coli* strain measured with the ELISA in sequential urine and serum samples from 15 patients with pyelonephritis and 7 with cystitis The antibody levels were expressed in per cent of reference samples a) IgA antibodies and b) IgG antibodies in the urine of the pyelonephritis patients, c) IgA antibodies and d) IgG antibodies in the serum from the pyelonephritis patients e) IgA antibodies and f) IgG antibodies in the urine from the patients with cystitis ● = girls △ = boys

urine samples Concentration techniques however cause degradation and denaturation of the antibodies (8) These problems can be eliminated by employing the enzyme linked immunosorbent assay ELISA (3) that is sufficiently sensitive to quantitate antibodies in unconcentrated urine samples (12) Furthermore the ELISA has the advantage of measuring separately and with similar efficiency (1) the antibodies of the different immunoglobulin classes including secretory IgA

In a previous preliminary study we have reported urinary antibodies appearing during UTI using the ELISA (12) The present investigation was aimed to give a more detailed picture of the urine and serum antibody response in sequences of samples from children with infections at different levels of the urinary tract We wanted to analyse the urine antibodies with special reference to their appearance in relation to serum antibodies their time relation to the onset of the infection and the possiblity that they at least partly could be locally produced Finally we wanted to determine if the antibodies in the urine samples which are more easily available than serum particularly from infants and children could be used for differentiation between upper and lower UTI

MATERIAL AND METHODS

Patients Twentytwo infants and children with significant bacteriuria ($\geq 10^5$ bacteria/ml) caused by *Escherichia coli* presenting at the Childrens Hospital Goteborg were included in the study Out of these 13 were girls with acute symptomatic pyelonephritis *i e* with fever exceeding 38 5° C and fulfilling at least two of the following criteria transiently decreased renal concentrating capacity in relation to age increased erythrocyte sedimentation rate ESR (>25 mm/h with the micro method) or increased C reactive protein CRP (>20 mg/l) (13) Nine of these 13 patients (age 8 months to 8 years) had their first known attack of pyelonephritis and 4 patients (age 4 to 10 years) had recurrences with previous infections between 10 and 30 months earlier Three girls observed with their first known attack of acute pyelonephritis recurred within one month with a second UTI caused by bacteria of the same serotype as during their first infection These infections were also included in this study In addition two boys 1 and 2 months old having their first known attack of pyelonephritis were investigated Suprapubic bladder puncture was performed on these young patients to obtain urine for bacterial cultures

Seven girls age 2 to 16 years had acute cystitis 4 had their first known UTI and 3 had recurrent attacks The criteria for the diagnosis of cystitis were symptoms of burning and frequency absence of loin pain temperature not exceeding 38 0° C and normal concentration capacity ESR and/or CRP level

In all patients with UTI the first day with fever or other symptoms was taken as the onset of infection All the patients were treated for 10 days with pivampicillin (50 mg/kg day) or sulphafurazol (200 mg/kg day) according to the antibiogram

Intravenous pyelography (IVP) and micturating cystourethrography (MCU) was performed on all patients with acute pyelonephritis No obstructions were recorded but 5 patients had vesicoureteric reflux to the renal pelvis but without any dilatation Parenchymal reduction was seen in one of these cases Only 2 patients with acute cystitis were radiologically examined and in both the results were normal Consecutive urine and serum samples were collected from all children during the follow up All samples were stored at −20° C without any addition of preservative The urines were centrifuged at 2000 *g* for 10 minutes before analysis

Antigens The *E coli* isolates from the patients were O grouped by direct bacterial agglutination employing specific antisera (20) Strains spontaneously agglutinating in saline were designated OR

A pool of strains of the 8 *E coli* groups most common in UTI was used as antigens for the references in the ELISA (14) The urine and serum samples from each patient were analysed against the O antigen of the infecting strain Somatic O antigens were prepared by heat extraction from the *E coli* urinary isolates and from the 8 strains mentioned above (10) The determination of the serotypes of the K antigens was performed as described by *Kayser* (16)

Antibody determinations The samples were investigated for O antigen specific antibodies of the IgG IgA and IgM classes as well as for secretory component carrying antibodies secretory IgA (SIgA) The antibodies were recorded with the enzyme linked immunosorbent assay ELISA (3) as earlier described (1 14) The alkaline phosphatase (Sigma Chemical Co St Louis USA) used for conjugation with the anti immunoglobulins had a specific activity of 1140 U/mg protein

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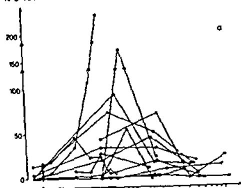
Creatinine analysis The creatinine concentrations of the urine samples were determined using a Technicon® Auto Analyzer (Technicon Instrument Co Ltd Chertsey Surrey Great Britain)

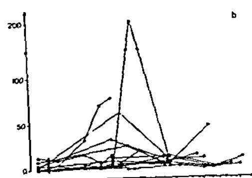
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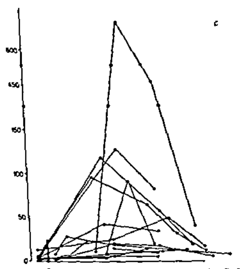
RESULTS

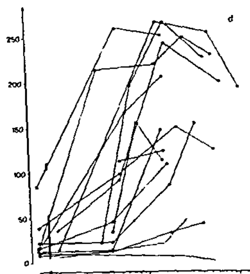
Patients with Acute Pyelonephritis

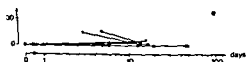
Urinary antibodies Antibodies of the IgG and IgA classes as well as antibodies carrying SC were detected in the urine samples from all patients with pyelonephritis The antibody levels obtained with the anti IgA conjugate parallelled those obtained with the anti SC conjugate but due to too few samples from each patient no statistical evaluation











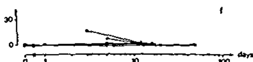


Fig 1 IgA and [illegible]
sequential urine
expressed [illegible]

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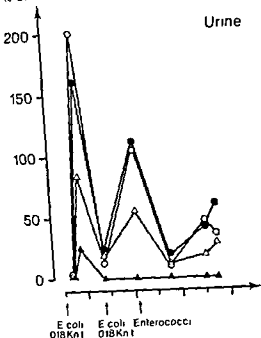
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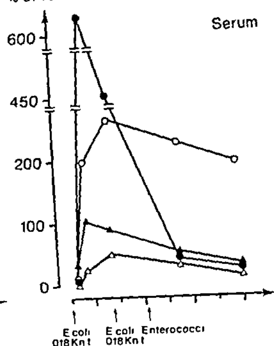
RESULTS

Patients with Acute Pyelonephritis

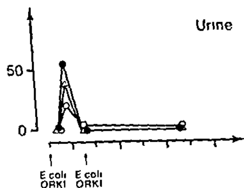
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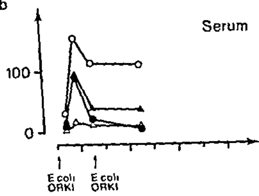
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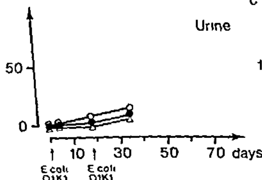


b





c



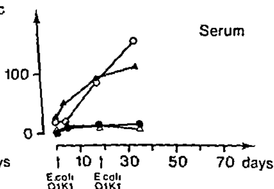


Fig 2 Urine and serum antibody levels against the O antigen of the infecting *E coli* strains expressed as per cent of reference samples in 3 patients (a–c) with recurrences of UTI Antibodies detected with anti IgG = ○ anti IgA = ● anti SC = △ and anti IgM = ▲ The arrows indicate the time of the first and recurrent infections K n t = K antigen not testable

TABLE I *Antibody Levels in Urine and Serum from Patients with Acute Pyelonephritis and Cystitis The Antibody Levels were Measured against Somatic Antigen Prepared from the Infecting* E coli *strains and were Expressed as per Cent of References Mean and (Range) of Highest Values Encountered in the Patients are Given*

			Urine antibodies				Serum antibodies			
Diagnosis	Sex	n	IgG	IgA	SIgA	IgM	IgG	IgA	SIgA	IgM
Pyelonephritis	F	13	41 (6–201)	64 (3–221)	37 (11–87)	6 (0–26)	183 (44–266)	97 (6–632)	21 (0–91)	93 (46–155)
Pyelonephritis	M	2	8 (5–10)	6 (2–10)	10 (8–11)	0	30 (11–49)	12 (0–24)	0	0
Cystitis	F	7	4 (0–17)	6 (0–16)	10 (0–24)	0	23 (7–49)	10 (5–16)	1 (0–8)	43 (26–68)

could be performed The antibodies carrying SC were obviously of the SIgA type, since most urines contained no IgM antibodies

Increases of the urine antibodies to the O antigen of the patient s strain were seen during the course of infection in most of the patients with pyelonephritis The IgA antibody levels in the urines increased in 10 of the 13 girls but not in the 2 infant boys (Fig 1a) Similar raises in urine IgG antibody levels were seen in 7 of the 13 girls (Fig 1b) No increased urine IgA or IgG antibody levels were noted in 2 of the girls and the 2 infant boys In only 4 patients IgM antibodies could occasionally be recorded

The urine antibody levels of any immunoglobulin class measured in the girls with first and with recurrent pyelonephritis did not differ ($p > 0.10$) as tested with the Wilcoxon rank sum test Therefore the results from these patients are presented together The means and ranges of the maximum antibody levels for the different immunoglobulin classes recorded in the girls with pyelonephritis are shown in Table 1 The antibody levels showed large individual differences but with increases in most patients In 3 girls the urine antibody levels were low in the range of those obtained in the patients with cystitis One of the strains causing the infections in these girls was spontaneously agglutinating in saline (OR) while the others belonged to O groups 1 and 18 The values of the girls with pyelonephritis still differed from those of the patients with cystitis ($p < 0.01$) as tested with the Wilcoxon rank sum test The infant boys with acute pyelonephritis differed from the girls and only had low levels of urinary IgG IgA and SIgA antibodies (Table 1 and Fig 1) without any pronounced increases

The 3 girls with pyelonephritis who became reinfected within one month showed varying antibody amounts in their urine (Figs 2a–c) Patient A had a pronounced response of IgG IgA and SIgA antibodies after the first infection When she recurred with an acute cystitis caused by an *E coli* strain of the same O group as in the first infection only low levels of urine antibodies could be detected When she attracted another attack of acute pyelonephritis caused by enterococci (Fig 2a) high antibody levels against the *E coli* strain causing the previous UTI s were recorded IgM antibodies were found in the urine of patient A but only after the first acute pyelonephritis In patient B only a moderate antibody response was seen and only after the first UTI (Fig 2b) whereas no such urine antibody response could be detected in patient C (Fig 2c)

Antibodies of the IgA and IgG classes against the infecting bacteria appeared at the same time in relation to the onset of infection in the urine samples from 9 of the girls and the 2 boys with acute pyelonephritis In the urines from the other 4 girls the IgA antibodies were detected after the IgG antibodies The IgA and IgG antibodies reached their maximum levels on the same day in 9 of the girls with pyelonephritis In 2 patients the IgA antibodies reached their maxima before the IgG antibodies and in another 2 patients the opposite pattern was seen The highest levels of IgA antibodies against the infecting *E coli* strain appeared within 10 days after onset of the infection in the urine from 8 of 13 girls The urine antibodies did not originate from the plasma as indicated by the lack of correlation between the urine antibody levels and the creatinine concentrations in the urines ($p > 0.10$) as tested with the Spearman rank correlation test

The presence of bacteria in the urine samples particularly during the first days after initiation of treatment may result in falsely low urine antibody values because of absorption of urine antibodies It was found that an addition of 10^6–10^7 bacteria/ml urine decreased the urine antibody levels by approximately 50 per cent (6)

Much of the antibodies recorded in the urine may be locally produced in the urinary tract Thus SIgA antibodies appeared in the urine from some of the patients with pyelonephritis and most of the patients with cystitis before such antibodies could be detected in the serum Furthermore there was a good correlation between the antibody levels determined with the anti IgA and the anti-SC conjugates indicating that much of the IgA antibodies was of the secretory type which is produced locally (7) For the IgG antibodies however the present study does not give any information as to whether or not they were locally produced in the urinary tract Such a local production of some of the IgG antibodies was suggested [illegible]

[illegible]

locally synthesized in the kidneys of rabbits with experimental *E coli* pyelonephritis (22 23)

In an experimental model using rats local immunization in the bladder seems to protect against ascending pyelonephritis via urine antibodies (17) Such a protective role of the antibodies in the urine from humans has not been definitely established but the differences in several characteristics of the bacteria in patients with untreated ABU compared to those causing acute cystitis or pyelonephritis may be the result of a selection pressure caused by urine antibodies resulting in »antigenic drift« (5 21) One possible important mode of protection by urine antibodies is suggested by the observation by *Svanborg Edén & Svennerholm* that [illegible]

[illegible]

The urine antibody levels recorded with the ELISA can not be used as a single diagnostic measure to differentiate pyelonephritis from cystitis patients since in 2 of the 13 girls with pyelonephritis no raised antibody levels were seen The observation that the serum and urine antibodies could not be used diagnostically in small infants with acute pyelonephritis agrees with an earlier report of their deficient serum antibody response (9) Together with other criteria (12) however, the findings of increased urine antibody levels may help in the diagnosis of UTI with differentiation between upper and lower infection

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The skilful technical assistance of *Lena Beijer* is very much appreciated This study was supported by grants from the *Medical Faculty of the University of Goteborg* the *First of May Flower Fund* the *Swedish Medical Research Council* (project no 215) and the *Ellen Walter and Lennart Hesselman Foundation for Scientific Research*

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Serum antibodies and their relation to urine antibodies The serum antibody levels increased in all patients with pyelonephritis except in one of the 2 infant boys The serum antibody values in the girls with first and those with recurrent attacks showed no statistically significant differences ($p>0$ 10) as tested with the Wilcoxon rank sum test so these patients were evaluated together The highest levels of IgG antibodies in the girls appeared about 2 weeks after the onset of the infection and the IgA SIgA and IgM antibody levels reached their maxima after 1 to 2 weeks Increases in the serum IgA antibody levels were found in 9 of the girls and in 1 boy as shown in Fig 1c In 9 girls IgA antibodies appeared before SIgA antibodies in the serum and in 3 they were detected in the same serum sample One girl lacked detectable serum SIgA antibodies The SIgA antibodies were found within 10 days after the onset of the infection in the serum from 10 of 13 girls The IgG antibody levels rose in all except one boy (Fig 1d) and the IgM antibody levels increased in 8 girls In the 2 infant boys no serum IgM antibodies were recorded The means and ranges of the maximum serum antibody levels are given in Table 1 The antibody values showed large individual variation but were significantly different from those obtained in patients with cystitis ($p<0$ 01) as tested with the Wilcoxon rank sum test

The serum antibody patterns for the 3 girls with recurrences within one month are shown in Figs 2a–c High levels of the IgG IgA and IgM amtibodies were recorded in patient A after the first infection (Fig 2a) The SIgA levels also rose In patient B IgG IgA and IgM antibody responses were recorded after the first infection only The SIgA antibodies increased slightly (Fig 2b) In patient C the antibody increase was slow and only the IgG and IgM antibody levels rose (Fig 2c)

Serum SIgA antibodies were detected before urine SIgA antibodies in 3 of the girls with pyelonephritis Another 5 patients had SIgA antibodies detectable on the same day in the serum and urine and in 3 patients serum antibodies of the SIgA type appeared after the urine SIgA antibodies (In 2 patients interpretation was impossible due to differences in the timing of sampling) In the 2 boys SIgA antibodies could not be recorded in the serum either during the infection or follow up period but such antibodies were found in the urine Serum IgG antibodies were detected either before or on the same day as urine IgG antibodies In no patient did serum IgG antibodies appear after urine antibodies (In 6 patients the difference in sampling of the serum and urine made interpretation of IgG antibody appearance impossible)

Patients with Acute Cystitis

Urinary antibodies Urine antibodies were detected in all except 2 patients with acute cystitis The levels of IgG IgA and SIgA antibodies were low throughout the study as illustrated for IgA and IgG antibodies in Figs 1e and 1f The antibody levels obtained in the patients with their first attack of acute cystitis did not differ from those in patients with recurrences The means and ranges of the antibody levels are given in Table 1 showing that some of the antibody values were as high as those found in a few patients with acute pyelonephritis The antibody levels in the group of patients with cystitis were however significantly lower than those recorded in the group of patients with pyelonephritis ($p<0$ 01) as tested with the Wilcoxon rank sum test

IgA and IgG antibodies were detected on the same day in the urine from 4 of the 7 patients with cystitis In one patient IgA antibodies appeared after IgG antibodies and in the remaining 2 patients no urine antibodies were recorded

Serum antibodies and their relation to urine antibodies Low IgG IgA and IgM serum antibody levels without any consistent increases were recorded in all patients with cystitis (Table 1) Antibodies of the secretory type could be detected in the serum from only one patient while these antibodies appeared in the urine from 5 patients One girl had serum SIgA antibodies in the absence of urine SIgA antibodies and one lacked both serum and urine SIgA antibodies All patients had serum IgG antibodies detectable either before or on the same day as urine IgG antibodies

DISCUSSION

The findings in the present study of high levels of IgG IgA and SIgA antibodies against the O antigen of the infecting strain in the urine from patients with pyelonephritis correspond to our earlier preliminary report (12) The urine antibody levels in the patients with acute cystitis were low compared to those found in patients with pyelonephritis They were also slightly lower than those previously reported in patients with asymptomatic bacteriuria ABU (5 12) but were increased in comparison to healthy children where low levels of IgG and occasionally IgA but not SIgA anti O antibodies are observed (12 *Sohl Åkerlund* unpublished results) The higher antibody levels found in the patients with ABU compared to those with cystitis may be explained by the ABU patients often carrying their bacteria for long periods possibly resulting in a stimulation of the immune system

Acta path microbiol scand Sect. C, 87 37–40, 1979

PRECIPITATION OF STREPTOCOCCAL PEPTIDOGLYCAN BY HUMAN SERA: INFLUENCE OF ANTI-IMMUNOGLOBULINS

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The Institute of Medical Microbiology, University of Lund, Solvegatan 23, S-223 62 Lund Sweden

Schalen C & Christensen P Precipitation of streptococcal peptidoglycan by human sera Influence of anti immunoglobulins Acta path microbiol scand Sect. C, 87 37–40, 1979

Antibodies to streptococcal peptidoglycan (PG) were detected by gel precipitation in 38% of sera from blood donors and in 71% of sera with a Waaler Rose test titre of ≥1 64 Twenty-six rheumatoid arthritis sera revealed patterns of interference with complete or partial fusion between PG and aggregated human IgG while none of the sera precipitating both these preparations showed non-interference The reactions were interpreted as denoting interference between the PG antibody complexes and aggregated IgG Conversion of some non precipitating blood donor sera to PG precipitation was obtained by addition of isolated rheumatoid factor in itself not precipitating PG, to the sera Thus the high frequency of PG precipitation among rheumatoid arthritis sera could – at least in part – be attributed to the participation of anti IgG in the reaction

Key words Streptococci anti immunoglobulins, peptidoglycan identity reactions

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The peptidoglycan (PG) constitutes the rigid part of the bacterial cell wall and is a heteropolymer composed of glycan strands cross linked through short peptide side chains [illegible]

The biological properties of PG include activation of the complement system, cytotoxicity for mammalian cells and inhibition of phagocytosis of bacteria (for review see *Heymer* 1975) A possible role of PG in rheumatoid arthritis and other »autoimmune« diseases has been discussed on the basis of these effects and the finding that more rheumatoid arthritis sera than normal human sera precipitate streptococcal PG in double diffusion in gel analyses (*Braun & Holm* 1970, *Zitnan et al* 1970) The present investigation was performed in order to elucidate, if the presence of anti-Ig could account for the high frequency of PG-precipitation among rheumatoid arthritis sera

MATERIALS AND METHODS

Non-Dialysable PG

Group A streptococci type M1 (Colindale No 8198) were cultured in 5 litres Todd Hewitt broth at 37°C After centrifugation, the bacteria were extracted three times with formamide at 150°C (*Fuller* 1938) The residue containing PG, was washed three times in [illegible] glucosamine 0 032 µmol/mg Rhamnose was not detectable indicating the absence of group-specific carbohydrate One mg lyophilized PG/ml PBS was solubilized by sonication for 60 min After removal of the insoluble residue by centrifugation the PG was dialysed at 4°C for 18 h against an equal volume of PBS, and for another 24 h against 100 volumes of distilled water

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volumes) which caused six of 16 sera examined to precipitate PG The RF chosen did not in itself precipitate PG but precipitated aggIgG

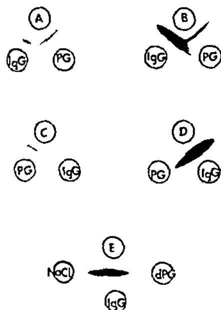


Fig 1 Immunodiffusion patterns between PG and aggIgG revealed by different RF positive sera A Serum giving Ouchterlony type I reaction B Serum giving Ouchterlony type III reaction C Serum only precipitating PG D Serum only precipitating aggIgG E Serum showing inhibition of the precipitation with aggIgG by dialysable PG Symbols IgG – aggIgG PG – non dialysable PG dPG – dialysable PG

TABLE 4 *The Effect of Dialysable PG on the Precipitation of aggIgG by RF Positive Sera*

Precipitation pattern against non-dialysable PG and aggIgG	No of sera tested	Dialysable PG	
		Inhibition	No inhibition
Type I (interference with complete fusion)	5	1	4
Type III (interference with partial fusion)	8	3	5
Sera precipitating aggIgG but not PG	7	3	4
Total no of sera	20	7	13

DISCUSSION

In the present study sera from healthy individuals precipitated PG in 38% *Zitnan et al* (1970) reported that 30% of such sera had antibodies to [illegible]

52% of normal human sera The failure of *Braun & Holm* (1970) to detect PG precipitating sera among 100 blood donors could partially be ascribed to variations in PG preparations or to differences among the populations studied

The results showed that more RF positive sera 71% of specimens tested precipitated PG than did normal sera which is in accordance with other reports *Zitnan et al* (1970) and *Braun & Holm* (1970) reported that 54% and 73% respectively, of rheumatoid arthritis sera precipitated PG Juvenile rheumatoid arthritis sera have been reported to contain more PG antibodies than normal sera in a study using a RIA technique (*Heymer et al* 1976)

Bokisch et al (1973) reported that two out of four sera from rabbits immunized with group C streptococci contained homogenous 7S anti IgG with reactivity for PG as well as the Fc portion of rabbit IgG The authors discussed the possibility of such double specificity of anti immunoglobulins in RF positive sera against the report of *Braun & Holm* (1970) Our present results show that sera containing RF precipitated both aggIgG and PG in higher frequencies than did blood donor sera However in a heterogeneous sample of RF negative patient sera such a correlation was not found between precipitation of PG and aggIgG Our findings that normal sera only exceptionally precipitated aggIgG although 38% precipitated PG and that several patient sera precipitated aggIgG but not PG also provided evidence against the existence of antibodies reactive with both IgG Fc and PG An immunological specificity of any antibody for the C-terminal sequence of the pentapeptide in PG as well as for the Fc portion of IgG is »difficult to reconcile from an immunochemical point of view« as stated by *Bokisch et al* (1973)

Our results indicated that the anti immunoglobulins of human sera do not *per se* interact with PG However the presence of anti Ig might be the possible cause of the more frequent precipitation of PG by RF positive sera than by normal sera It is known that anti Ig contributes to the high agglutination titres against several microorganisms often found in RF positive sera (see *Muller* 1962) For example the L-agglutination of streptococci by rheumatoid arthritis sera was shown to be influenced by anti IgG (*Christensen et al* 1975) The

Dialysable PG

A low molecular weight fraction of PG passing the membrane on dialysis of the solubilized preparation against an equal volume of PBS at 4°C for 18 h was used for some inhibition studies

Human Sera

Among the sera sent to our department for Waaler-Rose test (*Waaler* 1940) 72 rheumatoid factor (RF) positive and 94 RF negative sera were randomly selected The RF positive sera had a titre of ≥1 64, while the titres of the RF negative sera were ≤1 16, with the system used, a titre of ≥1 32 was considered positive In addition 50 sera were from healthy blood donors The sera were heated at 56°C for 30 min and stored at −20°C

Gel Filtration

A RF positive human serum was filtrated on a Sephadex G200 column (Pharmacia Fine Chemicals Uppsala Sweden) The fractions of the void volume capable to agglutinate latex particles (Hyland Lab Inc Costa Mesa Calif, U S A) were pooled and concentrated to yield the same Waaler Rose titre (1 256) as the original serum

Double Immunodiffusion-in Gel

Aggregated IgG (aggIgG) was prepared by sonication of heated IgG as described (*Schalen & Christensen* 1977) Five to seven µl of PG and of aggIgG 5 0–6 3 mg/ml PBS were used The sera 15–25 µl were used undiluted

* kindly performed by *Sigfrid Svensson* Ph D Dept of Clinical Chemistry, Lund

RESULTS

Proportions of Various Sera Precipitating Streptococcal PG and aggIgG

Normal sera precipitated non dialysable PG in 38% (Table 1) Two of them (4%) precipitated also aggIgG Seventy-one % of RF positive sera precipitated PG and 96% aggIgG Comparing

TABLE 1 *Frequency of Precipitation of PG and aggIgG*

	No of sera tested	Proportion of sera precipitating PG	aggIgG
Patient sera			
RF positive	72	71%	96%
RF negative	94	52%	15%
Blood donor sera	52	38%	4%

TABLE 2 *Precipitation of PG and aggIgG by RF Negative Patient Sera*

Precipitation of aggIgG	No of sera	No of sera precipitating PG
Yes	14	6 (43%)
No	80	43 (54%)
Total no of sera	94	49 (52%)

normal sera and RF positive sera more RF positive sera precipitated both preparations (p 0 01, Chi Square test)

RF negative patient sera precipitated PG to 52% and 15% precipitated aggIgG No correlation was found between presence of antibodies to aggIgG and capacity to precipitate PG (Table 2)

Diffusion-in-Gel Analysis of the Precipitation of PG and aggIgG by RF Positive Sera

Of 26 RF positive sera, Ouchterlony type I reaction (interference with complete fusion, *Ouchterlony & Nilsson* 1978) was noted for 5 The remaining 21 sera gave reactions of interference with partial fusion (type III) between PG and aggIgG Reactions of non-interference (type II) were not observed (Table 3 and Fig 1)

None of the sera precipitated dialysable PG However, dialysable PG inhibited the precipitation of aggIgG (Ouchterlony pattern type IV) for 7 out of 20 sera precipitating aggIgG (Fig 1) Of the 7 sera 3 did not precipitate the non-dialysable PG whereas one showed a pattern of interference with complete fusion and 3 with partial fusion between the precipitation lines for this PG and aggIgG (Table 4)

Addition of Isolated Rheumatoid Factor to Blood Donor Sera
Effect on Capacity to Precipitate PG

Rheumatoid factor (RF) preparation was added to blood donor sera not precipitating PG (equal

TABLE 3 *Immunodiffusion Patterns of RF Positive Sera Precipitating both PG and aggIgG*

Precipitation pattern against PG and aggIgG	No of sera
Type I (interference with complete fusion)	5
Type II (non interference)	0
Type III (interference with partial fusion)	21

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SALMONELLA TYPHI – INDUCED STIMULATION OF BLOOD LYMPHOCYTES FROM PERSONS WITH PREVIOUS TYPHOID FEVER

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Mogensen H H *Salmonella typhi* – induced stimulation of blood lymphocytes from persons with previous typhoid fever Acta path microbiol. scand. Sect. C 87 41–45 1979

In 21 persons with previous typhoid fever and in 15 controls thymidine incorporation of blood lymphocytes stimulated *in vitro* by killed *Salmonella typhi* was studied The optimal culture conditions were established to be a cell density of 10^5 cells per vial stimulated with 10^5 *S typhi* and incubated for 5 days The lymphocyte response to *S typhi* was significantly higher in the typhoid group than in the controls The lymphocyte responsiveness was not correlated to the time elapsed since the attack and was not found different in patients who had had typhoid relapse No difference was found in lymphocyte response to the mitogens PHA PWM and Con A between the typhoid group and the controls

Key words Lymphocytes *Salmonella typhi* cell mediated immunity typhoid fever

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The nature of immunity in typhoid fever, a disease unique to man is largely unknown It has been demonstrated that specific antibodies in blood are not correlated to the degree of protection (5) Antibodies in intestinal fluid could be important, but have so far not been [illegible]

establish the optimal conditions for lymphocyte transformation *in vitro* by *Salmonella typhi*-antigen 2 to compare lymphocyte responsiveness to *S typhi* in persons with previous typhoid fever and in healthy controls 3 to investigate whether any correlations exist between lymphocyte responsiveness to *S typhi* and certain clinical features Time past since typhoid fever and appearance of typhoid relapse

MATERIALS AND METHODS

Subjects Six males and 15 females aged 8–71 years (average 36 years) with actual or previous typhoid fever from the Department of Infectious Diseases Rigshospitalet were included The diagnosis was in all cases established by culture of *S typhi* from the stools and in 15 cases also from the blood Fifteen persons were treated with chloramphenicol, 2 g per day for 14 days 2 persons were treated with ampicillin and 4 did not receive any antibiotic treatment. In four of the patients typhoid relapse occurred (within two months) In the remaining 17 patients the clinical course was normal None became chronic carrier of *S typhi*

One of these persons was examined one and two months after the onset of disease (Chloramphenicol had been withdrawed 8 days prior to the first examination) The remaining 20 persons who were examined once each had had typhoid fever 3 months to 33 years previously

Seven persons had received TAB-vaccination one or several times before or after the disease and 10 were unvaccinated In 4 persons the vaccination status was unknown

participation of anti-Ig from rheumatoid arthritis sera in precipitation reactions by binding to antigen-antibody complexes has been demonstrated by several investigators (see *Muller* 1962) *Vaughan* (1956) and *Edelman et al* (1958) showed that RF precipitated soluble immune complexes, formed in antigen excess The participation of anti Ig in the precipitation of antigen by specific antibodies has been illustrated by the use of radiolabelled or fluorescein labelled RF by *Edelman et al* (1958) and some current methods for the demonstration of immune complexes are based on this RF reactivity (*Lurhuma et al* 1976) *Hamers et al* (1975) reported that anti-Ig s appearing in rabbits hyperimmunized with *Micrococcus lysodeicticus*, can reveal non precipitating allotype antigen-antibody systems Similarly, antibodies to PG in rheumatoid arthritis sera, occurring in amounts not detectable by immunodiffusion in-gel, might become detectable by the amplifying effect of simultaneously present anti-Ig s In our study, six of 16 blood donor sera not precipitating PG did so on addition of anti-IgG

The Ouchterlony patterns I and III obtained between PG and aggIgG should thus not be taken as an indication of immunological similarities between PG and aggIgG, according to our concept a more plausible interpretation is complete or partial interference between PG anti PG-antibody complexes and aggIgG

Edelman et al (1958) showed that RFs precipitated γ-globulin aggregates larger than 20S however, they also showed a reaction between RF and aggregates of lower molecular weight, which did not result in precipitation In accordance with these findings, dialysable PG might give small complexes of PG- anti PG antibodies which do not precipitate RF Such small complexes might explain the inhibition of the precipitation between aggIgG and RF positive sera obtained with dialysable PG

The present investigation did thus not support the view that the higher frequency of PG precipitation found among RF positive sera is caused by presence of a higher amount of specific antibodies to PG in such sera than in normal sera, the high frequency of precipitation could rather be explained as due to the presence of RF in the sera

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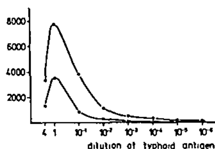


Fig 3 Thymidine uptake by lymphocytes after stimulation with varying doses of killed *S typhi* The lymphocytes were obtained from a patient one month (O——O) and two months (●——●) after the onset of disease Dilution of typhoid antigen 1 is equal to 10^8 killed *S typhi*

maximal response to this antigen preparation was in one case obtained with 10^6, in 5 cases with 10^7 and in 14 cases with 10^8 killed *S typhi* per 500 µl culture In 4 of these last persons a higher antigen concentration (2×10^8 or 4×10^8 *S typhi* per 500 µl) was tested giving a lower response than 10^8 *S t ph* In the con . . h

Comparison of Typhoid Group and Controls

Lymphocyte responses to different *S typhi* concentrations are shown in Table 1 In spite of a great variation, the responses obtained at all 4 antigen concentrations (and the maximal response) are significantly higher in the typhoid group than in the controls ($p < 0\ 001$, Wilcoxon rank sum test) The separation between the two groups is most marked at low antigen concentrations, although this trend was not statistically significant (Wilcoxon rank sum test)

The thymidine incorporation by unstimulated lymphocytes was higher in the typhoid group than in the control group, however, the difference is not significant ($p = 0\ 3$) and is mainly caused by two particularly high values

Correlation between Clinical Data and Lymphocyte Responsiveness

a) Lymphocyte responses in the 4 persons with typhoid relapse and in the 17 without were 4310 and 5079 c p m respectively (mean values) This difference is not significant (Wilcoxon rank sum test)

b) Time since typhoid fever The maximal lymphocyte response to killed *S typhi* was not correlated to the time elapsed since the attack of typhoid fever (Fig 4, Kendall rank sum test)

c) Since the groups were too inhomogeneous, it

TABLE 1 *Thymidine Uptake b L mphoc Ob ...*
Contr

		number of *S typhi* per 500 µl					maximal response
		0	10^5	10^6	10^7	10^8	
typhoid group	mean c p m (SD)	302 (456)	1589 (1918)	2496 (1995)	3617 (2411)	4923 (2988)	5009 (3003)
controls	mean c p m (SD)	114 (84)	146 (282)	227 (240)	369 (541)	778 (828)	817 (842)
ratio of responses typhoid/controls			10 9/1	11 0/1	9 8/1	6 3/1	6 1/1

Fifteen healthy volunteers aged 22–66 years (average 36 years, 2 males and 13 females) served as controls They had not had typhoid fever and were all seronegative for *S typhi* 0 and H-antibodies None were TAB-vaccinated

All donors (except the patient who was examined during the acute stage) were free of any febrile disease at least four weeks prior to investigation

Lymphocyte culture 30–40 ml of blood were drawn in 20 ml of RPMI 1640 (Gibco Bio cult) with heparin under sterile conditions Lymphocytes plus monocytes were isolated on a Ficoll-Isopaque gradient (4) and washed three times in RPMI 1640 with 5 per cent pooled serum from young non-transfused males, and the cells were cultured in 500 µl RPMI with 15 per cent serum According to the results of cell titration experiments 10^5 cells per vial (38 × 12 5 mm, roundbottomed, NUNC, Roskilde) were employed in subsequent experiments According to the results of kinetic experiments the cells were cultured for 120 h (mitogenstimulated cultures for 72 h) To the medium were added penicillin 400 IU per ml), streptomycin (0 4 mg per ml) and glutamin (0 04 mM per ml) All cultures were set up in triplicate, and were incubated at 37 5° C in 5 % CO_2 in humidified air 0 05 µCi ^{14}C-thymidine (specific activity 55 2 mCi/mM, New England Nuclear) was added 24 hours before termination The cells were collected on glassfibre filters on a Skatron cell harvesting machine and the incorporated radioactivity was measured in a Beckmann liquid scintillation counter after addition of 2 ml of scintillation fluid (Insta-Gel Packard) to the filters The results are given as means of triplicate determinations in counts per minute (c p m) minus c p m in unstimulated cultures

Antigen preparation *S typhi* OH-preparation (phenol-killed bacteria, Behring Institute) was washed 3 times and resuspended in isotonic saline This preparation was employed at 4 different concentrations employing 10-fold dilutions from 10^8 to 10^5 bacteria per 500 µl The maximal response is defined as the highest value obtained by any of the varying antigen doses

Mitogen preparations The mitogens were each emplo-

... tack sol tion ...

tion test (kindly performed by ... Statens Seruminstitut, Copenhagen) A seronegative sample is defined as one with a titer below 50

RESULTS

Cell Density Requirement

The thymidine uptake expressed as c p m per cell is shown at 4 different cell densities (Fig 1, experiments were carried out in 4 persons with previous typhoid fever) Each of these cultures was stimulated with 10^7 killed *S typhi* The highest

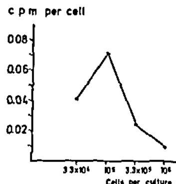


Fig 1 Thymidine incorporation expressed as counts per minute per cell at 4 different cell densities after stimulation with 10^7 killed *S typhi* The cell donor had had typhoid fever nine years previously

reponse per cell was in 1 case obtained at a cell density of 3 3 × 10^4 and in 3 cases at 10^5 cells per 500 µl originally cultured This cell density was employed in subsequent experiments

Kinetics of the Response

Cultures were stimulated with 10^7 killed *S typhi* and harvested after 4, 5, 6 and 7 days of culture Thymidine uptake was maximal on the sixth day of culture (Fig 2, similar results were obtained in 4 persons) In order to study the cultures during the period of exponential growth, a culture period of 5 days was employed in subsequent experiments

Optimal Dose of Antigen

In a patient with typhoid fever the lymphocyte response to varying doses of *S typhi* was determined at two occasions (Fig 3) The maximal response was at both occasions obtained with 10^8 *S typhi* In the persons with previous typhoid fever the

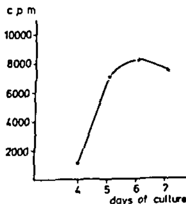


Fig 2 Thymidine incorporation by 10^5 lymphocytes from a person with previous typhoid fever, the cells were cultured for 4, 5, 6 and 7 days after stimulation with 10^7 killed *S typhi*

technical assistance Cand scient *Lars Ryder* is thanked for help in statistical calculations

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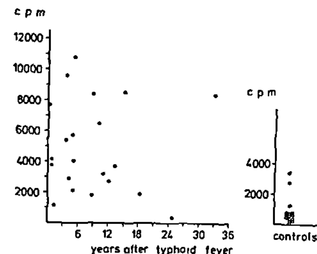
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[illegible]
●
with relapse O typhoid fever without relapse
Right the maximal response in 15 controls

was not possible to evaluate the possible correlation between lymphocyte responsiveness to the antibiotic treatment given, to the duration of fever or to the status of TAB-vaccination

Mitogenic Response

The lymphocyte responses to mitogens in the typhoid group and in the controls were (mean values ± SD) 21839 ± 6767 and 26216 ± 9799 c p m (PHA), 4365 ± 1778 and 4484 ± 1529 c p m (PWM), and 11740 ± 4917 and 12722 ± 3840 c p m (Con-A) respectively The differences are not significant (Wilcoxon rank sum test)

Serum Antibodies

Four persons were examined within 3 years after the attack of typhoid fever O and H titers were positive (titers 50 to 200) except for one H titer Seventeen persons were examined more than three years after their attack of typhoid fever these were all seronegative except for one case (O titer 50) A comparison of antibody titers to lymphocyte responsiveness was not possible since most titers were negative or low

DISCUSSION

In experimental infection in mice employing *Salmonella typhimurium* it has been demonstrated that immunity to this disease depends on antibodies in serum which after transfer to non immune mice protect these against infection (8) In contrast, resistance to typhoid fever in man appears not to depend on antibodies to O H or Vi antigens (5)

Cell-mediated immune responses in man to *S typhi* (lysed by ultrasonication) have previously been demonstrated by means of the leucocyte migration inhibition technique (2, 9), positive results were found significantly more often in patients with typhoid fever (68%) than in normal persons (40%) (9) The inhibition of leucocyte migration was demonstrable after the first week of illness in uncomplicated cases but in complicated cases it appeared later coinciding with clinical recovery

In the present study another test of cell mediated immunity *in vitro* the lymphocyte blast transformation assay, has demonstrated high responsiveness to typhoid antigen in lymphocytes obtained from persons with previous typhoid fever This is in agreement with the results of *Vylegzhanin & Bashirova* (1972), who demonstrated increased responsiveness of lymphocytes to typhoid antigen within the first six weeks of typhoid fever

The antigen preparation of killed bacteria employed in this study did elicit a higher lymphocyte response in persons with previous typhoid fever than in the controls (Table 1) In similar investigations of cell mediated immunity to *Pseudomonas aeruginosa* in cystic fibrosis and to *Neisseria meningitidis* in meningococcal meningitis heat killed bacterial antigen did not elicit a specific lymphocyte response (1, 7)

No definite limit can be drawn to separate the typhoid and the control group (Fig 4) A similar overlapping was found with the leucocyte migration inhibition test carried out in India (9) Since typhoid fever is endemic in India giving many subclinical infections some of the positive results in the controls could be explained this way A similar explanation is not probable in Denmark a more likely explanation may be based on crossreactions between antigens of *S typhi* and other bacteria as demonstrated in a wide variety of bacteria including *S typhi* by crossed immuno-electrophorectic analysis (6)

Besides many microbial preparations have a non antigen specific mitogenic effect (3 10) It will be important to investigate whether purifications of individual antigens for *in vitro* lymphocyte stimulation will allow a more specific response to be detected

Contrary to specific antibodies in blood which decrease within a few years after typhoid fever the lymphocyte responsiveness is long lasting (Fig 4) without significant decrease

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THE TIMING OF THE IMMUNE RESPONSE IN RELATION TO VIRUS GROWTH DETERMINES THE OUTCOME OF THE LCM INFECTION

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Randrup Thomsen A Volkert, M & Marker O The timing of the immune response in relation to virus growth determines *the outcome of the LCM infection Acta path microbiol scand Sect C 87* 47–54 1979

In the present study earlier observations of a dual role of the immune response against lymphocytic choriomeningitis (LCM) virus were confirmed and extended At different times after intracerebral (i c) inoculation of the virus groups of immunosuppressed recipients were transplanted with primary affe... blood of untransplanted ... id so were the brains of ... challenge The results ... a definite correlation between the brain virus titre at the time of the immune attack and the clinical outcome of the LCM infection Furthermore the results indicated that the extent of the extraneural infection is not unimportant but may affect the outcome of the infection through competition for the effector cells The possible implications of these findings are discussed

Key words LCM infection, cell mediated immunity immune response timing virus growth

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Inoculated intracerebrally (i c.) into adult mice lymphocytic choriomeningitis (LCM) virus replicates rapidly in the neural membranes and lethal ...

... 23) While B cells seem to be of no importance in the acute disease (14) many experiments have shown T cells to be central to the pathogenesis of this syndrome (3 4 11 13 23) Moreover recent observations strongly indicate that a short lived T cell population appearing transiently during the acute infection and rich in cytotoxic cells is the key factor in the events leading to the death of the host animal (5 27) However the T cell response is also essential in controlling the infection (3 13 16 23) ... ion to acutely infected mice may also reduce the virus titre and may even save the lives of the recipients (13 20 25, 28)

To explain this dual role of the immune response the following hypothesis has been advanced by several investigators (13 21, 25) if the virus content of a delicate organ (such as the brain) has had time to rise above a certain level before the effector T cells are present in sufficient numbers extensive cell destruction and death will be the result If the virus titre is below this level destruction will be less and instead of death the result will be suppression of the infection In spite of the fact that all parties concerned seem to agree on this assumption few experiments have been carried out trying to define this critical level of virus multiplication the time after i c inoculation at which it is reached and its relation to the degree of cell mediated immunity (CMI) (17, 21)

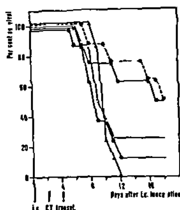


Fig 1 The capacity of various spleen cell populations to induce fatal disease in virus infected immunosuppressed syngeneic mice The recipients were given 10^3 LD_{50} of virus i c and dosed with 150 mg/kg of Cy after two days after further two days they were given 100×10^6 normal cells (■——■) day 7 immune cells (●——●) day 9 immune cells (O——O) memory cells (▲——▲) or received no further treatment (●– –●) Each group consisted of eight mice

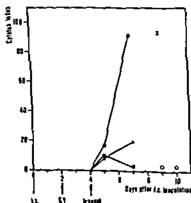


Fig 2 The time-course of cytotoxic activity in spleens of virus infected immunosuppressed syngeneic mice receiving day 9 immune cells (●——●) or day 9 immune cells pre irradiated with 600 R (▲——▲) or 900 R (■——■) The recipients were given 10^3 LD_{50} of virus i c and dosed with 150 mg/kg Cy two days later after further two days they were given 100×10^6 cells of the cell population in question or received no further treatment (O) Also included is untreated i p infected mice (×) sampled day 9 PI (peak cytotoxicity activity (19)) Effector target ratio was 25 1 The results are expressed as cytotoxic indices (18)

peak of CMI did not in our system have the rapid effect shown by *Doherty & Zinkernagel* (5) In an attempt to clarify the reasons for the delay observed in our system the following experiment was carried out Groups of i c infected recipients treated with Cy as described were transplanted on day 4 PI with either untreated day 9 immune cells or day 9 immune cells that had been irradiated *in vitro* with 600 or 900 R Twenty four hours and 3 days after transfer spleens of the transplanted mice were harvested and tested for cytotoxicity The experiment was carried out so that the results to be compared were obtained in the same cytotoxic assay Fig 2 shows that only few cytotoxic cells were found in the spleens of the recipients 24 hours after transfer of untreated day 9 immune cells whereas after 3 days the cytotoxicity had reached a level about that of untreated mice 9 days PI i e the level which is lethal to i c infected untreated mice It is also demonstrated that this increase in cytotoxicity from day 1 to day 3 after transfer was clearly sensitive to pre irradiation of the transferred cells with both 600 and 900 R It should be noted that no cytotoxicity was observed in the spleens of the Cy treated mice on day 9–10 PI when cytotoxic activity is normally maximal

The effect of transfer of primary effector cells and memory cells at different times after i c challenge The observation that the increase in CMI after transfer of day 9 immune cells occurred within only 3 days whereas the killing effect of these cells was postponed until day 5–6 emphasizes that factors other than the state of the CMI influence the outcome of the LCM infection In order to study this problem further primary effectors and memory cells were transferred to immunosuppressed i c infected recipients at different times PI However since the experiment described above clearly showed that in our system Cy treatment was not ideal for this purpose because of the high background lethality the immunosuppression was carried out with X irradiation in the following experiments

Groups of recipients treated with 275 R one day before i c inoculation were transplanted with primary effectors or memory cells on different days as stated in Table 1 The results are presented as the cumulated mortality over an observation period of 10 days after transfer (Table 1) This period was preferred since no LCM specific deaths were observed later than nine days after transplantation In Table 1 are also shown the results from one typical cytotoxic assay carried out with the cell populations used in these experiments

The data presented show as an general pattern that all three cell populations caused a high mortality when the cell transplantation was carried

The aim of the present study was therefore to investigate systematically the interplay between the factors a priori expected to influence the outcome of LCM virus infection in mice the virus content of the brain the virus content of other organs possibly competing for the effector cells and the state of the CMI For this purpose cell populations representing different states of immunity were transferred to immunosuppressed recipients at various times after i c virus challenge In this way various combinations of CMI and virus concentration were obtained and could be studied

MATERIALS AND METHODS

Virus The LCM virus strain used throughout the study was originally obtained from Dr Traub (Ludwig Maximillians Universitat West Germany) and has been used in this laboratory for many years It is kept at −70°C as a 10 per cent spleen suspension from infected mice When necessary it is passed in mice in recent years exclusively in C3H mice For infection of target L cells in the cytotoxic test virus propagated in L cells was used (19)

Virus titration Virus titration were carried out by i c inoculations into ordinary 12–14 g white Swiss mice When the virus content of organs or blood was determined individual titrations were carried out as described previously (17) Titration end points were calculated by the Karber method (15) and titres were expressed as log 10 LD_{50}/0 03 ml intracerebral dose It should be mentioned that the Traub strain is lethal to adult mice only if the virus is inoculated i c If other routes of infection are employed no disease is produced

Mice Except for titration purposes highly inbred adult (2–4 months) C3H mice (C3H/Ssc 1) of both sexes were used throughout the study

Immunosuppressed recipients Two methods of immunosuppression were employed (1) Cyclophosphamide and (2) X irradiation The cyclophosphamide (Cy Endoxan Asta® ASTA Werke West Germany) was given intraperitoneally (i p) in a dose of 150 mg/kg to mice infected i c with $10^3 LD_{50}$ of LCM virus two days previously In our system this treatment prevented acute LCM disease but during a period of about three weeks after the infection practically all mice died with signs of general wasting

The X irradiation was administered by a Siemens Stabilipan therapy machine operated with the following factors 200 kV 15 mA 1 0 mm copper filtration The dose rate was 47 R/min and half value layer was 1 5 mm of copper The dose given was 275 R and in all experiments the irradiation was carried out one day before i c inoculation with $10^3 LD_{50}$ of virus This treatment was chosen because earlier experiments in this laboratory (2) as well as recent preliminary experiments had shown it to give optimal survival During the second week after the infection all mice treated this way developed general signs of sickness and some of them displayed mild neurological symptoms (tonic-clonic convulsions on tail spinning) around day 7–11 PI but only a few mice (<10%) died with typical LCM symptoms However also this treatment resulted in late deaths with signs of general wasting and anaemia but only in a small proportion of the animals

Transplantation of syngenic immune cells Lymphoid cells representing primary effectors were harvested from the spleens of mice infected i p with $10^3 LD_{50}$ of virus 7 or 9 days previously (representing just prepeak and peak cytotoxic activity (19)) Lymphoid cells representing memory cells were harvested from mothers of infected babies about 2–3 months after the babies were born Such mice have undergone a natural infection and are solidly immune (24)

The cell suspensions were prepared as described earlier (19) except that only spleen cells were used Transplantations were always carried out by the intravenous route (i v) and between animals of the same sex the dose given was 100×10^6 cells in 0 5 ml

Cell mediated immunity (CMI) The CMI was determined as the *in vitro* cytotoxicity of single cell suspensions of spleen cells upon syngenic LCM virus infected L cells The cytotoxicity was expressed as a cytotoxic index or as lytic units (LU 50) per 10^6 spleen cells The test and the calculations were carried out as described in a recent paper (18)

RESULTS

Induction of fatal LCM in transplanted Cy treated recipients Experiments carried out in different laboratories by the use of the Armstrong strain of the LCM virus have shown that both primary effectors and memory cells have the ability to kill immunosuppressed i c infected mice if transferred to these early (4–5 days) after i c inoculation (5 11 13)

In order to investigate if this was also the case when the Traub strain of the virus and C3H mice were employed the following experiment was set up Groups of 8 i c infected mice treated with Cy as described were used as recipients Four days after the inoculation two days after the Cy treatment normal cells primary effector cells or memory cells were transferred to these recipients The mortality was registered daily over a period of 14 days after the transplantation and is shown in Fig 1 It is seen that all three population containing pre sensitized cells showed almost the same pattern killing a large proportion (75–100%) of the host animals in average 5–6 days after the transfer In contrast mice receiving normal cells and untransplanted controls for the main part died somewhat later

Cytotoxicity in Cy treated recipients after transfer of day 9 immune cells While the results obtained with memory cells confirmed previously recorded results (11 13) primary effectors harvested at the

Blood virus titre of mice surviving early transfer of immune cells It has been suggested that the protective effect of an early immune response is due to the antiviral effect of the effector T cells (13, 25) To establish whether this could explain why most mice survived cell transfer in the very early phase of infection the blood virus titre of mice that had received primary effectors or memory cells on the day of infection was compared on day 7 PI to that of untransplanted controls It was found that transfer of any of the immune cell populations caused a clearcut reduction of the blood virus titre on the day chosen (mean $\leqq 10^{0.8}$ compared to mean $= 10^{2.4}$); normal cells had no effect on the viremia (mean $= 10^{2.5}$)

The time-course of virus concentration in the brain of mice receiving day 9 immune cells at different times PI To further analyse the events determining the outcome of the LCM infection, the following experiment was carried out Four groups of recipients were given day 9 immune cells on days 0 2 4 and 7 PI, respectively At different times after cell transfer four mice from each group were killed and the virus content of their brains was titrated

Fig 4 demonstrates that when the recipients were transplanted on the day of i c inoculation, viral replication in the brain was initiated and went on undisturbed for about 3–4 days Then the spread of infection was aborted and in the next few days all

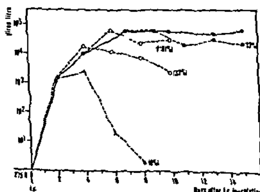


Fig 4 The time-course of brain virus titre in pre irradiated i c infected mice receiving day 9 immune cells on day 0 PI (△--△) day 2 PI (○--○) day 4 PI (□--□) or day 7 PI (■--■) The recipients were irradiated with 275 R one day before i c inoculation of 10^3 LD_{50} of virus and were given 100×10^6 day 9 immune cells on the days indicated or received no further treatment (●——●) Each point represents mean titre of four mice In *parentheses are shown* the mortality of the recipients this is the same as given in Table 1

the virus was cleared from the brain In contrast, when the cell transplant was given on day 4 PI, there was no influence on the virus replication which closely parallelled that of the untransplanted controls, and most recipients died about 4–8 days after the cell transfer Transfer of day 9 immune cells on day 2 PI gave intermediate results Furthermore, if the cell transfer was postponed until day 7 PI, the brain virus titre which at that time was maximal remained undisturbedly high but no deaths occurred

DISCUSSION

As mentioned in the introduction, it appears that the striking differences between the *in vivo* manifestations of the CMI response can be ascribed to the different outcomes of a race between the virus replication in the neural membranes, on the one hand, and the production of effector T cells on the other In the present study this hypothesis was tested further by transplanting i c infected, immunosuppressed recipients with the same cell populations at various times relative to the virus inoculation and comparing the mortality Three different cell populations were chosen for this purpose prepeak primary effector cells, peak primary effector cells, and memory cells It was found that during the first few days of the infection (day 0–4 PI) any of the cell populations employed caused the mortality to increase with increasing interval between the i c challenge and the cell transfer, and that this increase could be correlated to a similar increase in the brain virus titre (Table 1, Fig 3) Furthermore, a comparison between the results obtained with different cell populations reveals that the time at which the mortality increases after transfer of a given cell population is correlated to the effector activity as measured *in vitro* i e the higher the effector activity of the cell population, the later it can be transferred without causing high mortality Both of these observations add further strong support to the hypothesis that the timing of the immune response in relation to the virus growth in the brain is an essential *factor in* determining the outcome of the LCM infection It should be added that the expected residual immunological activity of the X-irradiated recipients can *hardly explain* the findings since the experiment using Cy treated recipients gave results that with regard to both mortality and MST were very similar to those obtained with X-irradiated mice on the same day, although the *Cy-treated* mice are almost totally immunosuppressed at the time when most recipients die (Figs 1 & 2)

TABLE I *Mortality in Immunosuppressed i c Infected Recipients Given Primary Effectors or Memory Cells at various Times Relative to the Virus Inoculation*[a]

Donor lymphocytes	Cytotoxicity in vitro[b]	Mortality after transfer on various days PI[c]					
		Day 0	Day 1	Day 2	Day 3	Day 4	Day 7
Memory cells	≤0 5	11/33	8/8	16/16	8/8	16/16	13/16
Day 7 immune cells	3 5	0/16	1/7	14/16	8/8	14/16	0/8
Day 9 immune cells	8 0	0/14	0/8	5/16	14/16	13/16	2/16
Normal cells	–	1/14	–	4/16	–	5/16	1/8
–		7/95	9/95	12/95	13/95	15/95	23/95

a Adult C3H mice pre irradiated with 275 R on day −1, given 10^3 LD_{50} of virus i c on day 0 and subsequently transplanted with 100×10^6 spleen cells from immune or non immune donors on the days stated

b Expressed in lytic units per 10^6 cells

c Proportion of mice dying during a 10 day observation period

out on day 3–4 PI, whereas both earlier and later transfer to some degree resulted in a lower mortality, this was particularly pronounced with the cell populations obtained during the primary response (day 7 and day 9 immune cells) which are rich in cytotoxic effector cells

The mean survival time (MST) was calculated in all instances in which a significant proportion of the recipients (≧ 1/3) were killed by the transplanted cells This was always found to be about 5–6 days and in no case was it possible to conclude that a given MST was significantly different from that observed when the same cell population was transplanted on other days or when other cell populations were transplanted on the same day

Virus concentration in the recipient mice The above reported results indicate as expected that the extent of the virus infection at the time of the immune attack plays a crucial role in determining the clinical outcome of the LCM infection In order to get a better picture of the correlation between the extent of the infection and the clinical outcome the following experiment was carried out

Groups of 6 mice pre irradiated and infected as described were killed at various intervals after i c inoculation and the virus content of their brains and blood was titrated the results appear from Fig 3 It is seen that the virus titre in the brain was steadily increasing from day 0 to day 7 PI when a maximum level of approx $10^{4.8}$ was reached and the titre remained at this level for the rest of the observation period It is also demonstrated that when the Traub strain of the virus is employed the increase in brain virus titre is followed closely by an almost parallel increase in viremia from day 0 to day 7–10 PI From then on the titre seems to flutter around a level of approx $10^{2.7}$, with a possibly decreasing tendency In all titration experiments day 18 (not shown) was the first day on which spontaneous clearing of viremia was observed in some mice

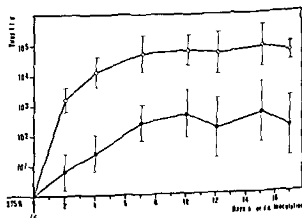


Fig 3 The time course of blood (●——●) and brain (○——○) virus titre in adult mice irradiated with 275 R one day before i c inoculation of 10^3 LD_{50} of virus Each point represents mean titre ± SD of six mice

beneficial to the host depending on the relative kinetics of virus replication and immune induction is to assume that it is the same immunological mechanism which is underlying both cytopathology and virus clearance Actually recent *in vitro* experiments carried out with vaccinia virus do suggest that the antiviral effect of T cells is accomplished by a direct cell mediated destruction of cells displaying viral antigen during the eclipse phase of the infectious cycle i e before infectious progeny is assembled (26) If this mechanism plays any role *in vivo* the cell destruction caused by the cytotoxic cells have found its teleological explanation

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How the apparently protective effect of an early immune response is accomplished is suggested by the finding that primary as well as secondary effector T cells have the capacity to reduce the virus titre in many organs (6 20) In accordance with this we found that all mice surviving early transfer of immune cells showed no more than trace levels of virus in their blood Moreover it appears from Fig 4 that if day 9 immune cells (peak primary effectors) are transferred to the immunosuppressed recipients on the day of infection the virus replication in the brain is aborted prematurely there are no deaths and the virus appears to be cleared as a direct result of the transferred cells With increasing interval between i c challenge and cell transfer the virus replication is allowed to take place unrestrictedly for a longer period and mortality increases When the interval is extended to 4 days the virus replication in the brain remains completely undisturbed and practically all the recipients die On the basis of these results it seems reasonable to conclude that the early presence of a CMI response protects the animal through a suppression of the infection in the brain before it reaches an extent which would make this organ too vulnerable to the immune attack

It is somewhat surprising that transfer of a cell population having maximal cytotoxic activity *in vitro* and almost immediate antiviral effect in the spleen and liver (19 20) does not as shown in Fig 4 cause any demonstrable decrease of the virus replication in the brain of the recipients until (3)–4 days after the transfer However this delay is probably due to purely mechanical factors which may slow down the race of the effectors to reach the infected target cells in the neural membranes

The correlation between mortality and brain virus titre at the time of the CMI response could theoretically be explained in two ways First that the number of infected cells in the neural membranes has to exceed a certain percentage of the total number before the immune attack causes essential damage Second that with the extension of the infection some especially vital area becomes infected and in this way susceptible to the immune attack However immunofluorescent studies have clearly demonstrated that during the early phase of i c infection in immunosuppressed adult mice the virus replicates predominantly in the neural membranes (16) Moreover that it is the number of antigen presenting cells – and not their distribution – which changes during the first few days thus clearly favouring the former of the two possible explanations

However the data presented in this paper allow us to go further than this and to draw some conclusions about the extent of virus infection which would constitute a lethal target for the immune attack If mice are transplanted with day 9 immune cells two days after i c challenge approximately one third of the recipients are killed whereas if the cell transplantation is postponed but one day practically all the recipients are killed (Table 1) The brain virus titre of mice receiving day 9 immune cells on day 2 PI shows that in this situation the effector T cells do not reduce virus multiplication until day (5)–6 PI (Fig 4) From the drastic increase in mortality which is found when the virus is allowed to multiply unrestrictedly for just one more day we find it reasonable to deduce that the level of infection found in untransplanted mice around day 5–6 PI may represent the approximate limit of virus concentration in the brain determining the outcome of the LCM infection This assumption is supported by the finding that memory cells which seem to need about 5–6 days to obtain effector capacities (7 9) are killing only one third of the recipients (MST = 5 5 days) if transferred on the day of infection whereas a later transfer leads to 100% mortality Moreover no deaths in any experimental group were ever observed earlier than day 5 PI indicating again that conditions for fatal disease are not present before that day

A situation totally different from the one discussed in the above arises if the cell transfer is postponed until day 7 PI From the blood virus titre curve presented in Fig 3 and in view of the findings of several authors (11 13 16) it seems probable that what has happened is that because of the immunosuppression the infection has spread to many organs so that the brain is no longer the main target for the immune attack Under these circumstances the clinical outcome of the infection will depend upon whether enough effector T cells are being generated to overcome the competition from other sites of infection and establish a sufficiently concentrated immune attack on the brain This would certainly explain why only a memory subset with its inherently greater potential for proliferation and/or differentiation (7 8 13 18 25) can induce fatal disease if the cell transfer is carried out on that day whereas mice receiving primary effectors will not only survive but also continue to carry infectious virus in their brains Competition from extraneural sites of infection may also explain the finding that in contrast to others using the Armstrong strain of the virus (5) which causes more localized infection we did not on any day observe primary effectors to have a more rapid killing effect than memory cells

The simplest explanation of the fact that the immune response may be detrimental as well as

beneficial to the host depending on the relative kinetics of virus replication and immune induction is to assume that it is the same immunological mechanism which is underlying both cytopathology and virus clearance Actually recent *in vitro* experiments carried out with vaccinia virus do suggest that the antiviral effect of T cells is accomplished by a direct cell mediated destruction of cells displaying viral antigen during the eclipse phase of the infectious cycle i e before infectious progeny is assembled (26) If this mechanism plays any role *in vivo* the cell destruction caused by the cytotoxic cells have found its teleological explanation

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GUINEA PIGS INBRED FOR STUDIES OF RESPIRATORY ANAPHYLAXIS

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Lundberg Lise Guinea pigs inbred for studies of respiratory anaphylaxis Acta path microbiol scand Sect C 87 55–66 1979

A selective inbreeding of approximately 24 generations of albino guinea pigs by brother × sister mating has resulted in two strains registered IMM/S and IMM/R with high and low responsiveness respectively to ovalbumin induced respiratory anaphylaxis The two guinea pig strains differed in their ability to be immunized by the inhalation of antigen and produce antibodies as well as to develop respiratory anaphylaxis A correlation between the strength of the anaphylactic reactions and the amount of hemagglutinating antibodies produced was observed When immunization was carried out by an intradermal injection of ovalbumin (OA) even in small doses incorporated in FCA guinea pigs from both strains produced hemagglutinating antibodies in nearly the same amount These antibodies do not influence the ability of the animals to react with a high respectively low anaphylactic response on subsequent challenge by inhalation of OA neither in the actively sensitized animals nor in passively sensitized animals However with repeated inhalations of OA desensitization occurred in the intradermally immunized high responders while the passively immunized high responders could be provoked several times without any signs of desensitization No systematical differences between the two strains with regard to sensitivity to inhalations of histamine were demonstrated The low responders were found to be less resistant to infections than high responders

Key words Selective inbreeding respiratory anaphylaxis active passive immunization high low responder

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The guinea pig has been the traditional immunologic research model of antigen induced airway obstruction The use of outbred guinea pigs in experimental work with respiratory anaphylaxis is impeded however because of variations in responsiveness within sex age and season of testing and also due to the large differences in individual responsiveness

In our own experiments relationships between methods of immunization antibody production and immediate hypersensitivity reactions could not be evaluated due to the dispersion in the results which was too large for drawing any statistically significant conclusion The larger ...

develop guinea pigs with more homogeneous immunological characteristics

Results from experiments with inbred strains of mice have shown that the immune response is under genetic control (8 15) In addition short term selective inbreeding of guinea pigs has demonstrated the possibility of separating the properties of good and poor production of antitoxin against diphteria anatoxin (27)

This report describes the development of two strains of guinea pigs with reproducible responses to inhalation of ovalbumin (OA) one strain with a high and one with a low anaphylactic response These strains were compared as regards their ability to develop hypersensitivity their susceptibility to respiratory anaphylaxis and their resistance to infections

MATERIALS AND METHODS

Antigen Ovalbumin crystallized three times obtained from I C N Pharmaceuticals Inc

Adjuvant Freund s Complete Adjuvant (FCA) containing 0 5 mg/ml *Mycobacterium tuberculosis* kindly supplied by the Tuberculin Department Statens Serum institut Copenhagen

Immunization by inhalation The following technique is used both in the initial immunization and in the *bronchial provocation test of anaphylaxis* The guinea pigs are placed in a box divided into individual sections by rough wire mesh A glass cover enables observation of the animals during the inhalation procedure A De Vilbiss nebulizer No 40 fitted in one side of the box introduces an aerosolized 0 5% antigen in saline solution at a pressure of 70 mm Hg The animals are exposed to antigen inhalation for 50 min once a week for 6 weeks 6–8 ml antigen solution are used during each inhalation Up to 30 animals can be exposed to antigen at the same time The severity of the anaphylactic reactions is evaluated during the inhalations and graded with 0–5 + according to the following scheme

Evaluation of the anaphylactic reactions in the bronchial provocation test

0	*No signs of anaphylactic reactions* – controls
+	Incidental or slightly forced respiration
+ +	Clearly forced respiration
+ + +	Strongly forced respiration fits of coughing
+ + + +	As 3 + but in addition the animal is *shocked and its general condition is poor*
+ + + + +	As 4 + but in addition an interruption in inhalation for treatment with aerosolized adrenaline is necessary

Intradermal Immunization The guinea pigs are immunized once by intradermal injections at 4 sites of the abdomen with 0 1 ml of saline solution of antigen emulsified in FCA

Passive immunization 1–3 ml samples of whole serum from guinea pigs actively immunized as described above are used for passive immunization The serum is injected into the recipient animal intraperitoneally 48 hours before antigen challenge

Bleedings One week after the last exposure to inhalation of antigen or 5 weeks after the intradermal immunization blood samples are collected Samples of sera are kept in the refrigerator at –20° C until testing

Antibody titration A quantitative analysis of the antibodies is carried out by a passive hemagglutination technique (3) Serial twofold dilutions of unheated serum are made beginning with a dilution of 1 20 All titrations are run with a standard serum containing a determined amount of antibody and a normal serum as control The titer is recorded as the number of tubes showing a positive hemagglutination

Measuring anaphylaxis The bronchial provocation test of anaphylaxis is carried out as described under the inhalation technique *The Schultz Dale test* the in vitro measuring of anaphylaxis is carried out as earlier described on isolated pieces of ileum (17) The anaphylactic response of the ileum is expressed as the lowest amount of antigen in mg added to the reaction chamber which generates a weak contraction

RESULTS

A Selection for breeding Albino guinea pigs were chosen for the experiments due to their suitability for cutaneous testing and marking Animals used to start the inbreeding were initially obtained from an outbred colony maintained at Statens Seruminstitut in Copenhagen from 1933

Three month-old guinea pigs of both sexes were exposed to repeated inhalations of aerosolized solutions of OA Some of the animals developed severe respiratory anaphylaxis and produced high titers of hemagglutinating antibodies while some showed no anaphylactic reactions and produced no measurable amounts of antibodies (Table 1)

Guinea pigs showing the strongest anaphylactic responses were bred together as were those showing no signs of anaphylaxis The offspring were tested when 2–4 months old and were selected for further breeding in the same manner as the parents The inbreeding was performed as brother × sister mating

B Problems during the breeding experiments Passive transfer of antibodies from mother to offspring sometimes caused very strong anaphylactic reactions in connection with the first antigen exposure of the young ones This passively transferred hypersensitivity could be observed in young ones from the first five litters which indicated that antibody transfer occurred over a year after the mother s last exposure to antigen These anaphylactic reactions often decreased with repeated antigen exposure sometimes resulting in a total loss of anaphylactic response and antibody production Even some guinea pigs which did not react on the first inhalation could develop this refractory state

Delaying the antigen exposure until after weaning was not possible since the ability of the animals to be sensitized by inhalation decreased with age as seen in Fig 1

A system in which the breeding animals were not exposed to antigen but were selected on the basis of antigen testing of brothers and sisters was abandoned after six generations of brother × sister mating because of poorer results with regard to homogeneity in immune response

C Results of the inbreeding experiments Results of the inhalation experiments on the original outbred colony (Table 1) show the large variations

TABLE I *Development of Respiratory Anaphylaxis by Repeated Inhalations of Aerosolized Aqueous Ovalbumin (OA) in Outbred 3 Months Old Female Guinea Pigs*

Weight	Response to OA inhalations						Hemagglut titer[a])	Schultz Dale test[b]) mg OA
	Day 0	Day 7	Day 14	Day 21	Day 28	Day 35	day 42	day 60
500	0	+	0	+ +	0	0	0	–C[c])
585	0	0	+ +	0	0	0	0	–C
510	0	0	0	0	0	0	4	–C
530	0	+ +	+	+ + +	+ +	+	3	0 1
440	0	0	0	+ +	+ + +	+	3	0 005
445	0	0	0	+ + +	0	0	4	0 005
425	0	0	0	+ +	+ + +	+	5	0 02
465	0	0	0	+ + +	+ + +	+	5	0 1
450	0	0	0	+ + + +	+ + + +	+ +	6	0 001
400	0	0	0	+ + + +	+ + + +	+ + +	6	0 0002
600	0	0	0	+ + + +	+ +	0	7	0 0005
465	0	0	+ + + +	+ + +	+	+	7	0 001
450	0	0	+ + +	+ + +	+ + +	0	8	0 0005
465	0	0	+ + + +	+ + + +	+ +	+ + +	8	0 0002
365	0	0	+ + + +	+ + +	+ + +	+ +	8	0 0005
650	0	0	+ + + +	+ + +	+ + +	0	9	0 001
500	0	0	+ + + +	+ + +	+ + +	0	9	0 0002
475	0	0	0	+ + + +	+ + + +	+	9	0 01
415	0	0	+ + + + +	+ + + +	+ + + +	+ +	9	0 0002
440	0	0	+ + + +	+ + +	+ + +	+ + +	10	0 0001

a) Standard serum titer 15
b) See Materials & Methods
c) –C – no contraction with 5 mg OA



Fig 1 Results of 6 weekly inhalations of aerosolized aqueous OA of all guinea pigs from the first four years of inbreeding at different ages
The numbers heading the columns indicate numbers of animals tested

No anaphylactic reaction
Mean anaphyl. reaction
Severe anaphyl reaction

♂ ♀

%
100
80
60
40
20
0

86 31 34 37 35 18 19 77 73 79 15 49 88 37 24 38 46 21 16 70 65 78 24 53

J F M A M J JL A S O N D J F M A M J JL A S O N D

Month of the 1st inhalation

Fig 2 Results of 6 weekly inhalations of aerosolized aqueous OA of all 2–4 month old guinea pigs from the first 4 years of inbreeding at different times of the year
The numbers heading the columns indicate numbers of animals tested

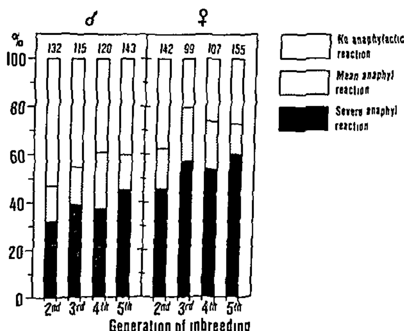


Fig 3 Results of 6 weekly inhalations of aerosolized aqueous OA of 2–5 month old guinea pigs evaluated for high anaphylactic reactivity in the first 5 generations of inbreeding
The numbers heading the columns indicate numbers of animals tested

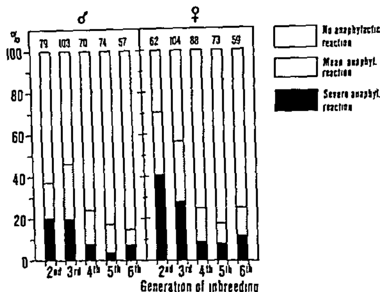


Fig 4 Results of 6 weekly inhalations of aerosolized aqueous OA of 2–5 month-old guinea pigs evaluated for low anaphylactic reactivity in the first 6 generations of inbreeding
The numbers heading the columns indiate numbers of animals tested

in immune response due to individual differences, and Figs 1 and 2 illustrate the variations observed in respiratory anaphylactic reactivity within age, sex and season

The results of inbreeding guinea pigs selected for high and low respiratory anaphylactic reactivity in the first 5–6 generations are shown in Figs 3 and 4 respectively The figures demonstrate the gradually developed separation of the two properties, when the original method was used

These results indicated that the sensitivity and resistance to inhalations of OA were hereditary by nature and based on this assumption the inbreeding was continued

D The resulting strains Today we have two strains of high responders 209 and 740, registered as IMM/S (7) inbred for 24 generations, and two strains of low responders 201 and 203 registered as IMM/R (7) inbred for 24 and 16 generations respectively

After 15 generations of selected inbreeding by the

tions of aerosolized aqueous OA, without variations due to sex or season

E Cross-breeding In an attempt at eliminating contemporary inbreeding of undesirable characteristics such as failing fertility, blindness, deformity of the forelegs and increased sensitivity to infections, and at the same time to preserve the already obtained immunological properties we have cross bred animals from two lines of 209 after 5–6 generations, and developed substrain 209y Likewise, we have crossbred animals from 201 with animals from 203, and developed substrain 201–03

The substrains 209y and 201–03 were then brother × sister mated for 16 and 12 generations respectively and after only two generations these substrains were already pure high- and low responder strains respectively

F The susceptibility to infections resulting primarily in pneumonia, differed significantly be tween high- and low responders, the low responders being less resistant to infections Table 2 shows the mortality of young guinea pigs in the different strains Table 3 shows th

died within the first six months ($\chi^2 = 30.6$, $p < 0.001$)

G Correlation between hemagglutinating antibodies and respiratory anaphylactic response This relationship demonstrated in the original animals

No anaphylactic reaction
Mean anaphyl reaction
Severe anaphyl reaction

♂ ♀

%	J	F	M	A	M	J	JL	A	S	O	N	D	J	F	M	A	M	J	JL	A	S	O	N	D
n	86	31	34	37	35	18	19	77	73	79	15	49	88	37	24	38	46	21	16	70	66	78	24	53

Month of the 1st inhalation

Fig 2 Results of 6 weekly inhalations of aerosolized aqueous OA of all 2–4 month old guinea pigs from the first 4 years of inbreeding at different times of the year
The numbers heading the columns indicate numbers of animals tested

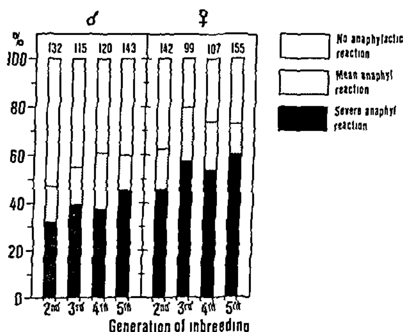


Fig 3 Results of 6 weekly *inhalations of aerosolized aqueous OA of 2–5 month* old guinea pigs evaluated for high anaphylactic reactivity in the first 5 generations of inbreeding
The numbers heading the columns indicate numbers of animals tested

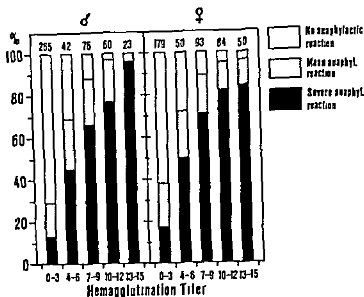


Fig 5 Correlation between respiratory anaphylaxis and hemagglutinating antibodies in high responders from the first 4 years of inbreeding (standard serum titer = 15)
The numbers heading the columns indicate numbers of animals tested

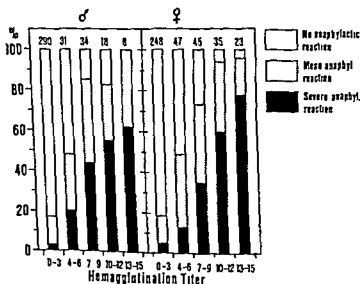


Fig 6 Correlation between respiratory anaphylaxis and hemagglutinating antibodies in low responders from the first 4 years of inbreeding (standard serum titer = 15)
The numbers heading the columns indicate numbers of animals tested

TABLE 2 *Stillbirth and Early Death in Various Substrains of Guinea Pigs Bred Through the Last Year (Stillbirth Includes Abortions of which Only a few were Observed)*

Strain/ substrain	Total No	Stillbirths No	%	Deaths 0–2 months No	%	Deaths 2–6 months No	%	Survivals No	%
IMM/S 209	211	7	3.3	7	3.3	2	1.0	195	92.4
IMM/S 209y	146	6	4.1	14	9.6	10	6.9	116	87.1
IMM/S 740	148	1	0.7	4	2.7	10	6.8	133	89.9
IMM/R 201	383	25	6.5	30	7.8	38	9.9	290	75.7
IMM/R 203	78	7	9.0	10	12.8	3	3.9	58	74.4
IMM/R 201–03	167	7	4.2	9	5.4	22	13.2	129	77.3

(Table 1), was maintained during the inbreeding, when the immunization was performed by inhalation (Figs 5 and 6)

H Intradermal immunization When animals from both strains were injected intradermally with different doses (0.1 µg, 1 µg, 10 µg) of OA incorporated in FCA, both high- and low-responders produced hemagglutinating antibodies in nearly the same amount However, a subsequent challenge with inhalation of OA showed, that the sensitivity to this treatment was not really influenced by these antibodies By repeated inhalations a decline in severity of the respiratory anaphylaxis was demonstrated (Table 4)

I Passive immunization with transfer of antibodies produced by injection of OA in FCA, followed by inhalation experiments, gave results comparable to the results from the active, intradermally immunized high- and low-responders following the first inhalation However, with repeated inhalations, no decline in severity of the anaphylactic reactions in high-responders was observed, and only low titers of hemagglutinating antibodies were produced The results given in Table 5 show, in addition, that antibodies formed in high- as well as in low-responders after immunization with OA in FCA are equal able to produce respiratory anaphylaxis when passively transferred

J Difference in susceptibility to respiratory anaphylaxis demonstrated by high and low responder guinea pigs, immunized actively or passively, led to an examination of the sensitivity to inhalation of histamine No systematical difference was found between animals from the two strains

TABLE 3 *Litter Size in Various Substrains of Guinea Pigs Bred Through the Last Year*

ain/ bstrain	Total no of litters	No of guinea pigs in litter at birth: 1 No	1 %	2 No	2 %	3 No	3 %	4 No	4 %	5 No	5 %	6 No	6 %	7 No	7 %	Average litter size	
IM/S 209	67	3	4.5	11	16.4	28	41.8	23	34.3	2	3.0					3.2	3.0
IM/S 209y	44	2	4.6	8	18.2	13	29.5	18	40.9	3	6.8					3.3	
IM/S 740	53	10	18.9	13	24.5	19	35.9	9	17.0	2	3.8					2.6	
IM/R 201	118	10	8.5	28	23.7	45	38.1	24	20.3	9	7.6	1	0.9	1	0.9	3.0	2.9
IM/R 203	28	2	7.1	7	25.0	14	50.0	2	7.1	2	7.1	1	3.6			2.9	
IM/R 201–03	61	6	9.8	22	36.1	24	39.3	7	11.5	2	3.3					2.6	

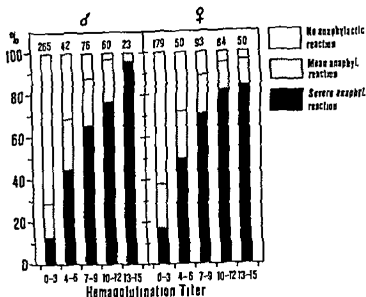


Fig 5 Correlation between respiratory anaphylaxis and hemagglutinating antibodies in high responders from the first 4 years of inbreeding (standard serum titer = 15)
The numbers heading the columns indicate numbers of animals tested

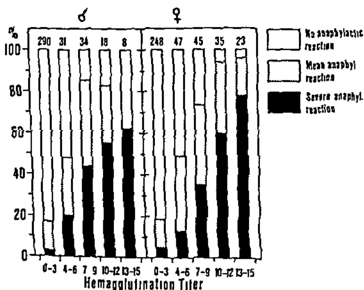


Fig 6 Correlation between respiratory anaphylaxis and hemagglutinating antibodies in low responders from the first 4 years of inbreeding (standard serum titer = 15)
The numbers heading the columns indicate numbers of animals tested

TABLE 4 *Respiratory Anaphylaxis in Strain IMM/R and IMM/S Guinea Pigs Actively Immunized with Ovalbumin (OA) Incorporated in Freund s Complete Adjuvant (FCA)*

Strain	OA + FCA µg Day 0	Cutaneous testing[a)] 0 1 µg OA Day 30	Hemagglutination titer[b)] Day 37	Inhalation of OA Day 50	Day 57	Day 64	Day 71	Day 78	Hemagglutination titer[b)] Day 85
IMM/R	10	15 mm	16	0	0	0	0	0	16
	10	14 mm	14	0	+ +	+ + +	+ +	+	13
	10	14 mm	14	0	0	+ + +	+ +	+	14
	10	14 mm	16	0	0	0	0	0	15
	1	14 mm	14	0	+ + + +	+	+	0	14
	1	13 mm	11	0	+ +	0	0	0	15
	1	14 mm	14	0	+	+ +	0	0	14
	1	13 mm	15	0	+	0	0	0	15
	1	12 mm	16	0	+	+	0	0	16
	1	13 mm	17	0	0	+	0	0	18
	0	2 mm	0	0	0	0	0	0	1
	0	3 mm	0	0	0	0	0	0	1
	0	not tested	0	0	0	0	0	0	0
	0	» »	0	0	0	0	0	0	0
	0	» »	0	0	0	0	0	0	0
IMM/S	1	15 mm	15	+ + + +	+ +	+ +	+ +	+ + +	13
	1	14 mm	13	+ + + + +	+ + + + +	+ + + + +	+ + + +	+	14
	10	17 mm	11	+ + +	+ + + +	+ + +	+ +	+ +	11
	10	15 mm	12	+ + + +	+ + + +	+ + + +	+ + +	+	12
	1	18 mm	17	+ + +	+ + + +	+	+	0	16
	1	13 mm	14	+ + + +	+	+	0	0	14
	1	20 mm	16	+ + + +	+ + + +	+ +	+ +	0	16
	1	12 mm	15	+ + +	+ + +	+	0	+ + + +	16
	1	16 mm	11	+ + + + +	+ + +	+ +	+	0	14
	1	14 mm	15	+ + + + +	+ + +	+ +	+ +	0	14
	1	16 mm	10	+ + + +	+ + + +	+	0	0	14
	1	14 mm	15	+ + + +	+ + + +	+ + + +	+ + +	+ + + +	13
	0	2 mm	9	+ + + + +	+ + + + +	No further inhalations becauce of severity of reactions			
	0	not tested	0	0	0	+ + + + +	+ + + + +	+ + + + +	7
	0	» »	0	0	0	+ + + + +	+ + + + +	+ + + + +	9
	0	» »	0	0	0	+ + + + +	+ + + + +	+ + + + +	9

TABLE 5 *Passive Transfer of Respiratory Anaphylaxis to Guinea Pigs from Strains IMM/S and IMM/R by Serum from Either of the Two Strains*

DONOR	μg OA + FCA	H aggl titer[a]	RECIPIENT	ml serum i.p.	Day 2	min	Day 9	min	Day 16	min	Day 23	min	Day 30	min	H aggl titer[a] Day 37
IMM/R	10	10	IMM/R	3	+	18	+ +	20	0	–	0	–	0	–	1
–	10	10	IMM/S	3	+ + + + +	3	+ + + + +	1	+ + + + +	2	+ + + + +	2	+ + + + +	3	3
–	10	11	IMM/S	3	+ + + + +	1	+ + + + +	3	+ + + + +	2	+ + + +	4	+ + + + +	2	5
–	10	11	IMM/R	3	+ + +	10	+ + +	25	0	–	+ +	20	+	10	4
–	10	10	IMM/S	3	+ + + + +	3	+ + + + +	2	+ + + + +	2	+ + + + +	3	+ + + + +	5	1
–	10	10	IMM/R	3	+ +	12	+	20	0	–	0	–	0	–	4
–	10	8	IMM/S	3	+ + + + +	4	+ + + + +	1	+ + + + +	1	+ + + + +	1	+ + + + +	1	2
–	10	8	IMM/R	3	+ + +	10	+ +	15	+ + +	20	+	10	+	20	2
–	1	10	IMM/S	3	+ + + + +	1	+ + + + +	1	+ + + + +	1	+ + + + +	1	+ + + + +	1	3
–	1	10	IMM/R	3	+ + + +	8	+	12	+	20	+	20	0	–	3
–	10	9	IMM/S	1	+ + + + +	1	+ + + + +	1	+ + + + +	1	+ + + + +	1			3
–	10	9	IMM/R	1	0	–	0	–	0	–	0	–			0
IMM/S	10	10	IMM/S	3	+ + + + +	3	+ + + + +	2	+ + + + +	3	+ + + + +	3	+ + + + +	1	5
–	10	10	IMM/R	3	+ + + +	3	+ + +	15	0	–	0	–	0	–	3
–	10	10	IMM/S	3	+ + + + +	4	+ + + +	3	+ + + +	8	+ + + +	8	+ + + + +	4	4
–	10	10	IMM/R	3	+	16	+	20	0	–	0	–	0	–	2
–	10	11	IMM/S	3	+ + + + +	4	+ + + + +	3	+ + + + +	3	+ + + + +	1	+ + + + +	1	6
–	10	11	IMM/R	3	+ +	20	+ + + +	15	0	–	0	–	0	–	3
–	10	11	IMM/R	3	+ + + +	4	0	–	+ +	20	+ +	20	+	10	3
–	1	10	IMM/S	3	+ + + + +	3	+ + + + +	1	+ + + +	3	+ + + +	8	+ + + + +	3	4
–	1	10	IMM/S	3	+ + + + +	1	+ + + + +	1	+ + + + +	2	+ + + + +	4	+ + + + +	2	4
–	10	11	IMM/S	1	+ + + + +	1	+ + + + +	3	+ + + + +	2	+ + + +	18			2
–	10	11	IMM/R	1	0	–	0	–	0	–	0	–			0

a) H aggl = hemagglutination titer (standard serum titer = 14)

TABLE 6 *Antibody Production and Respiratory Anaphylactic Responsiveness in Guinea Pigs from Strains IMM/S and IMM/R Immunized with Ovalbumin by Different Methods*

Strain	Imunization method	Respiratory anaphylactic response 1st challenge	subsequent challenges	Antibody titer before challenge	after challenge
IMM/S	inhalation	high	high moderate	high	high
IMM/R	inhalation	low	low	low	low
IMM/S	i d + FCA	high moderate	moderate low	high	high
IMM/R	i d + FCA	moderate low	low	high	high
IMM/S	passive	high	high	low	low
IMM/R	passive	low	low	low	low

and furthermore very large variations in sensitivity to histamine were observed *in the same animal with* repeated inhalations

DISCUSSION

Results of selective inbreeding The high and low-responder strains of guinea pigs developed should *now be inbred after the 24 generations of brother* × sister mating and in accordance with the intrastrain *immunologic homogeneity obtained for the proper*ties examined The two strains are now considered sufficiently pure for the performance of crossing experiments with high and low responders for studies of the genetic control of the different properties Correlations between certain major histocompatibility complex genes immune responsiveness and susceptibility to infectious and allergic diseases in a number of species including man have been demonstrated and discussed by several workers (2 10 21 25 26)

Animals from the two strains differ 1) in their

1 Difference in development of hypersensitivity can be explained at two levels 1) the permeability of the respiratory tract mucosa and 2) the antibody production *A difference in permeability of the respiratory tract mucosa to OA* is the simplest way to explain differences in the anaphylactic responsiveness between high and low responders both in ability to be immunized and in susceptibility to respiratory anaphylaxis in actively as well as in passively immunized animals The demonstrated age related responsiveness to respiratory anaphylaxis may also be explained by a difference in permeability of the mucosa which has been observed to be inversely related to the maturity of the individual (4)

The transient or weak anaphylactic respiratory *responses sometimes observed in strongly immun*ized low responders however indicate that an absorption of ovalbumin through the mucous membranes takes place in low responders too Furthermore recent studies in man indicate a similar absorption of allergens in both allergic patients (during asymptomatic periods) and in normal individuals (12 19)

Antibody production and respiratory anaphylactic *responsiveness by the different immunization me*thods are briefly outlined in Table 6

The amount of different antibodies produced in guinea pigs is known to depend on antigen route of administration dose and adjuvant (6 11 23) The respiratory anaphylaxis developed after repeated inhalations of OA may be due to the prescence of one or more of the three homocytotropic antibodies IgG 1a IgG 1b and IgE identified at present in guinea pigs (23)

By inhalation of OA a different ability to produce hemagglutinating antibodies in the two strains and a correlation between the amount of hemagglutinating antibodies formed and the anaphylactic respiratory responsiveness was demonstrated These results can be parallelled to a correlation demonstrated between hemagglutinating antibodies and IgE in different strains of mice when the immunization was initiated with small doses of OA adsorbed on aluminium hydroxide (25)

The discrepancy observed between the anaphylactic response and the quantity of hemagglutinating antibodies in guinea pigs from the two strains immunized with OA in FCA or by passively transferred antibodies and the variation observed in desensitizing effect of repeated inhalations may partly be explained by the presence of immunoglob

characteristics

different susceptibility to respiratory anaphylaxis (28)

However the severe respiratory anaphylaxis in the passively sensitized high responders which can be reproduced several times without desensitization may offer a system suited for *in vivo* testing of pharmacological agents of interest in asthma bronchiale

2 *The different susceptibility to respiratory anaphylaxis* observed in high and low responders is not due to a corresponding difference in sensitivity to inhalations of histamine In contrast two strains of guinea pigs inbred with regard to high and low sensitivity respectively to inhalation of histamine when sensitized with antigen showed the same difference in anaphylactic responsiveness by subsequent challenge with inhalation of aerosolized antigen solution (29)

The different susceptibility to respiratory anaphylaxis may further be explained 1) by differences both in the total amount of histamine in the lung tissue and the amount available for release in anaphylaxis as demonstrated in the inbred guinea pig strains 2 and 13 (28) 2) by a different ability of cells to accept reaginic antibodies as demonstrated with human leucocytes *in vitro* (20) or 3) by differences in the biochemical processes that are controlling the mediator release from the cells after the antigen antibody interaction on the cells The histamine release is considered to be controlled by cellular enzymes and the level of cyclic AMP while the number of cell bound antibody molecules determines the concentration of the corresponding antigen required for triggering the cells for histamine release (16 18)

Referring to the very large difference in susceptibility to respiratory anaphylaxis between the two strains the animals are supposed to be well suited for further studies of the processes leading to mediator release in anaphylaxis

3 *The different susceptibility to infections* observed in the two strains seems to be related to the responsiveness to respiratory anaphylaxis The two resulting substrains from cross breeding either high or low responders which possess the same anaphylactic responsiveness as the main strains simultaneously showed the same susceptibility to infections in spite of the fewer generations of inbreeding The attempt to enhance the natural resistance by cross breeding has been unsuccessful Furthermore the inhalation experiments which were run on the guinea pigs when 2–6 months old did not cause an elevated mortality in high responders due to infections regardless of the severe respiratory anaphylaxis provoked repeatedly in all high responders

Requirement for an animal model for studies of atopic reactions Several investigators have discussed in detail the enormous complexity of events in the atopic reactions in man and the possibilities and limitations of using animal models since for safety reasons only limited studies can be done in atopic humans (1 4 5 12 13 18 22 24 29)

The preliminary results presented here indicate a utilization of the two strains of guinea pigs for the original purpose the development of an animal model for examination of respiratory anaphylaxis

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This work was started in collaboration with my former chief Professor *K A Jensen* to whom I owe a debt of gratitude for inspiration and support I am much indebted to my present chief Professor *M Volkert* for giving me the possibility of continuing these prolonged experiments The careful and reliable assistance of the personnel in our animal house is gratefully acknowledged Finally I wish to thank my present laboratory assistant *Charlotte Berthelsen* as a representative of several assistants from whom I have received valuable technical assistance

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EFFECT OF MYCOPLASMAS ON PHAGOCYTOSIS AND IMMUNOCOMPETENCE IN RATS

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Thomsen A C & Heron I Effect of mycoplasmas on phagocytosis and immunocompetence in rats Acta path microbiol scand Sect. C 87 67–71 1979

The purpose of this study was to evaluate some possible mechanisms by which mycoplasmas may facilitate a subsequent bacterial infection The effect of *M arthritidis M hominis* and *U urealyticum* on lymphocyte reactivity to mitogens subsequent antibody response and the ability of neutrophils to carry out phagocytosis was investigated *In vitro* large doses of *M arthritidis* and *M hominis* depressed the reactivity of lymphocytes and the phagocytic ability of neutrophils. *In vivo* inoculation of mycoplasmas had no effect on reactivity of lymphocytes from rats and antibody response to subsequently injected *E coli* was normal However peritoneal neutrophils from rats injected intraperitoneally with large doses of *M arthritidis* and *M Hominis* were invalidated in their ability to cause phagocytosis of *E coli U urealyticum* had no observable effect on lymphocytes and neutrophils *in vitro* and *in vivo*

Key words Phagocytosis immunocompetence mycoplasmas rats

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Mycoplasma hominis and *Ureaplasma urealyticum* are frequently isolated from the human urogenital tract but their relationship to diseases remains in most cases unclear However a possible pathogenic role of mycoplasmas is indicated by reports of a facilitating effect on simultaneously (6) and subsequent (6 10 14) bacterial inflammation Thus the dose of *Eschericia coli* sufficient for the induction of suppurative pyelonephritis in rats was decreased a hundredfold when preceded by inoculation with *Mycoplasma arthritidis* (14)

[illegible]

certain antigens was depressed (12) resulting in a decreased secondary *in vitro* production of humoral antibodies

Mycoplasmas have also been observed to interact [illegible]

The present study was undertaken to compare the effects of mycoplasmas *in vitro* and *in vivo* on T lymphocytes B lymphocytes and neutrophils as expressed by the reactivity of lymphocytes to mitogens, the production of humoral antibodies and the ability of neutrophils to cause phagocytosis

MATERIALS AND METHODS

Rats Specific pathogen free male rats of an inbred strain of Hooded Lister weight 200–300 g were used

Mycoplasmas The strains used were strain 158 P 10 of *M arthritidis* which has been proved to be pathogenic in ra (7) [illegible]

modified Shepard s medium (1) The cultures were harvested by centrifugation at 50 000 g for 1 hour and resuspended in normal saline to a concentration of approximately 10^{8} colony forming units per ml (c f u / ml) Mycoplasmas were inoculated intraperitoneally

intracardially or directly into the kidney, as described previously (14) Control rats were inoculated with normal saline

Bacteria Strain 04 44/41 of *E coli* isolated from a patient with inflammation of the urinary tract, was cultivated in brain-heart broth to provide approximately 10^7 c f u /ml This dose is pathogenic for rats (14)

Examination of lymphocyte reactivity Cultures of rat lymphocytes were prepared from cervical lymph node and spleen as described previously (8) In brief, 5×10^5 lymphocytes were cultured in 0 2 ml of medium in microtitre plates The medium used was RPMI 1640 (Gibco), supplemented with penicillin 100 I U /ml, 5 per cent normal rat serum and 5×10^{-5} M 2-mercaptoethanol DNA synthesis was quantified by the uptake of ^{14}C-labelled thymidine during the last 16 hours of a 96 hour incubation period at 37° C Cultures were harvested on glass fibre filters The radioactivity on the dried filters was measured in a scintillation spectrometer

T lymphocytes were stimulated by the addition of phytohemagglutinin (PHA) 0 5 μg ml^{-1}, and concanavalin A (con A), 10 μg ml^{-1}, and in the mixed lymphocyte culture (MLC) by the addition of equal numbers of irradiated (4500 rad) allogeneic lymphocytes B cells were stimulated by 20 μg ml^{-1} of lipopolysaccharide (LPS)

The *in vitro* effect of mycoplasmas on lymphocyte reactivity was studied in lymphocyte cultures Doses of 10^8, 10^6 and 10^5 c f u /ml of the three mycoplasma strains were added to the cultures before stimulation, and each test was performed in quadruplicate Controls included the addition of normal saline to cultures

The *in vivo* effect was studied in lymphocytes removed from 40 rats after inoculation of 10^8 c f u of the mycoplasma strains or of normal saline into 10 rats each Injection was performed into the heart peritoneum and kidney simultaneously The rats were euthanized in pairs at 1 8, 15 22 and 29 days post-inoculation Lymphocytes from the cervical lymph node and spleen were cultivated and stimulated as above but streptomycin (100 μg/ml) was added to the medium to prevent growth of mycoplasmas

Examination of ability to produce antibodies to E coli Nine rats were each inoculated with 10^8 c f u of mycoplasmas or normal saline Injection was performed into the heart peritoneum and kidney simultaneously in all 36 rats 10^7 *E coli* was inoculated intraveneously 7 and 14 days later Serum samples were drawn from the tail vein of the rats before inoculation with *E coli* and subsequently every second day

Antibodies to *E coli* were determined in duplicate by the indirect haemagglutination method (11) A 10 hour culture of *E coli* strain 04 44/41 was boiled harvested and used as antigen

Examination of the ability of neutrophilic leukocytes to cause phagocytosis For the *in vitro* phagocytosis assay peritoneal and peripheral blood neutrophils from 16 rats were examined The rats were inoculated intraperitoneally with 0 1 per cent glycogen in normal saline The peritoneal exudate harvested 24 hours later contained approximately 10^7 cells per ml which by differential counting was shown to consist of 95 per cent or more neutrophils Heparinized tail vein blood from the rats was used for the preparation of peripheral leukocytes The blood was mixed with one-fourth of its volume of 6 per cent dextran 70 (Pharmacia) and incubated for one hour at 37° C to sediment the erythrocytes The supernatant contained approximately 10^4 leukocytes per ml, 20 per cent of which were neutrophils The leukocyte suspensions from peritoneal exudate and peripheral blood were centrifuged at 180 *g* for 5 min and the deposited cells resuspended to a concentration of 10^6 cells per ml in Eagle's minimal essential medium (MEM) supplemented with five per cent (v/v) foetal calf serum and adjusted to pH 7 2 with sodium bicarbonate The mycoplasma strains were added to four suspensions of leukocytes to a ratio of 100 1 or 10 1 of mycoplasmas per neutrophil An equivalent volume of saline was added to four leukocyte suspensions The suspensions were incubated at 37° C for 30 min *E coli* suspended in MEM was then added to a ratio of 10 1 and 1 1 bacteria per neutrophil, and enumerated in the supernatant in five concurrent aliquots after 0,30 and 60 minutes of further incubation at 37° C

The *in vivo* effect of mycoplasmas on leukocyte phagocytic function was studied using the peritoneal and peripheral blood leukocytes from 32 rats Six groups of four rats each were inoculated intraperitoneally with *M arthritidis M hominis* or *U urealyticum* suspended in 0 1% glycogen at two concentrations 10^8 and 10^6 c f u Eight rats inoculated with saline containing 0 1% glycogen served as controls The peritoneal exudate from these rats contained 10^7 cells per ml, 95 per cent of which were neutrophils, and the blood contained 10^4 leukocytes per ml, 20% of which were neutrophils The leukocytes were harvested and resuspended in MEM as above Mycoplasmas were never recovered from samples of these suspensions *E coli* was added and enumerated as above

RESULTS

Effect of Mycoplasmas on Lymphocyte Reactivity

The addition *in vitro* of *M arthritidis* and *M hominis* in doses of 10^8 and 10^6 c f u /ml reproducibly inhibited both the thymidine incorporation in lymphocytes stimulated with mitogens (PHA conA and LPS) and the MLC response A representative example is shown in Fig 1 in which an almost total impairment took place in the uptake of ^{14}C-labelled thymidine in PHA and conA stimulated lymphocyte cultures with 10^6 c f u /ml added The addition of 10^5 c f u of *M arthritidis* and *M hominis* increased the mitogenic response slightly but the addition of *U urealyticum* or normal saline had no significant effect on the responses Similar results were obtained when lymphocytes from cervical lymph node or from the spleen were tested

In vivo the mycoplasmas did not reveal inhibi

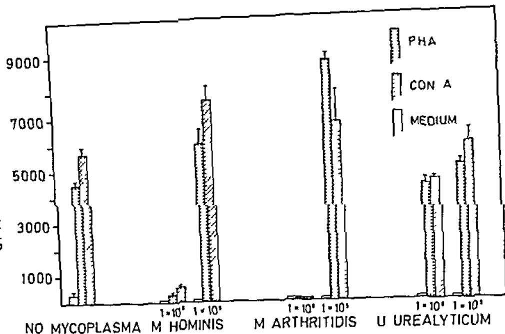


Fig 1 Reactivity of lymphocyte cul ures with *M arthr t d s M hom n s* or *U urealyt cum* added 10^6 or 10^5 c f u / ml mycoplasmas were added to rat lymphocyte cultures which were stimulated with PHA and con A

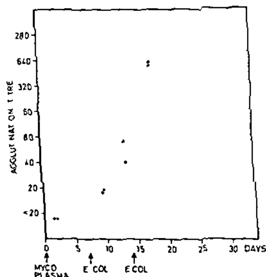


Fig Ant body response to *E col* in rats prev ously inoculated with 10^5 c f u of *M ar t r d s M hom nis* or *U urealyt cum*

Symbols refer to an ibod es to *E col* n rats previously inoculated with *M ar hr td s* (▲), *M hom n s* (O) *U ureal t cum* (■) or med um only (●)

tory effect Lymphocytes from both the cervical lymph node and the spleen of 30 rats removed during a period of 30 days after injection of *M arthritidis M hominis* or *U urealyticum* could all be stimulated by mitogens and in MLC to the same extent as lymphocytes from 10 control rats inoculated with saline Mycoplasmas were invari ably cultured from the lymph node and spleen of inoculated rats at the time of removal but lymphocyte cultures never yielded growth

Effect of Mycoplasmas on Antibody Formation to E coli

The *in vivo* formation of antibodies to *E coli* inoculated seven and 14 days after inoculation of mycoplasmas was naffected A b

urealyticum as compared with the nine controls previously inoculated with saline (Fig 2)

Effect of Mycoplasmas on the Phagocytosis of E coli *by Neutroph ls*

In vitro the peritoneal and peripheral blood neutrophils from eight rats were invalidated in their

TABLE 1. *The Effect of Mycoplasmas on Phagocytic Function of Peritoneal Neutrophils as Expressed by the Number of* E coli *after Mixing with the Neutrophils*

	Colony-forming units of *E coli* in suspension[a]					
	in vitro			*in vivo*		
Time of incubation (minutes) Mycoplasma species	0	30	60	0	30	60
M arthritidis	8×10^5	1×10^6	7×10^5	2×10^6	1×10^6	1×10^6
	1×10^6	1×10^6	2×10^6	9×10^5	7×10^5	7×10^5
M hominis	3×10^6	1×10^6	3×10^6	9×10^5	1×10^6	8×10^5
	7×10^5	9×10^5	8×10^5	2×10^6	2×10^6	2×10^6
U urealyticum	1×10^6	5×10^4	1×10^4	2×10^6	7×10^4	2×10^4
	1×10^6	1×10^4	2×10^4	9×10^5	5×10^4	5×10^4
No mycoplasmas	9×10^5	1×10^4	9×10^3	3×10^6	2×10^4	3×10^4
	7×10^5	2×10^4	2×10^4	8×10^5	9×10^3	1×10^4

[a] *Average of five concurrent enumerations of E coli added in the ratio of one bacteria to one leukocyte in two in vitro* and two *in vivo* experiments

ability to phagocytize *E coli* by *M arthritidis* and *M hominis* This was expressed by a constant number of *E coli* after incubation with neutrophils preincubated with the mycoplasmas (Table 1) The mycoplasmal inhibitory effect was independent of the ratios of mycoplasmas per leukocyte and bacteria per leukocyte tested Neutrophils from eight rats preincubated with *U urealyticum* or saline phagocytized *E coli*, resulting in a hundredfold decrease in the number of bacteria within one hour, regardless of the ratio of bacteria per leukocyte tested

In vivo, peritoneal neutrophils from eight rats inoculated with 10^8 c f u of *M arthritidis* or *M hominis* were invalidated in their ability to cause phagocytosis of *E coli*, as shown by the constant number of bacteria after mixing with the neutrophils (Table 1) Peripheral blood neutrophils, neutrophils from eight rats inoculated with 10^6 c f u of *M arthritidis* or *M hominis* and neutrophils from 16 rats with *U urealyticum* or saline, phagocytized *E coli* as expressed by a hundredfold decrease in the number of bacteria The leukocyte suspensions never yielded growth of mycoplasmas

DISCUSSION

The effect of mycoplasmas on lymphocyte function appears to be complex *In vitro* large doses of *M arthritidis* and *M hominis* inhibited thymidine incorporation in cultures where lymphocytes were stimulated by either B or T cell stimulating agents, while small doses increased the response We assume that these changes reflect decreased and increased DNA synthesis of the lymphocytes respectively, although this may not necessarily be the case Similar dosedependent decreasing and increasing effects of the two mycoplasma species have been observed in mice lymphocyte cultures (3), and a suppressive effect of large doses of both organisms on human lymphocytes has also been reported (12)

Furthermore, a decreased response of sensitized lymphocytes *in vitro* to tuberculin and alloantigens and impaired secondary *in vitro* production of antibodies to diphtheria toxoid has been demonstrated in lymphocyte cultures infected with *M arthritidis* or *M hominis* (12)

The suppressive effect of mycoplasmas on lymphocyte reactivity *in vitro* seems to be due to arginine depletion of the medium (3, 12), and is only expressed by arginine utilizing species, e g *M arthritidis* and *M hominis* (12), and not by species independent of arginine, e g *U urealyticum* as shown in the present study On the other hand the mitogenic properties have been observed for both glucose and arginine utilizing species, though as regards the latter only when added in small doses which do not deplete the arginine from the medium (3)

In vivo T and B lymphocytes from rats inoculated with mycoplasmas reacted normally to mitogens and allogeneic cells The B cell production of antibodies to *E coli* injected one and two weeks after the mycoplasma inoculation was also preserved This is in accordance with the observation that antibody response to sheep erythrocytes is unaffected by prior infection with arginine utilizing mycoplasmas (12) Thus where bacterial infection follows mycoplasmal infection the functions of T and B lymphocytes *in vivo* seem to be unaffected possibly due to the inability of mycoplasmas to depress markedly the levels of arginine in the host.

However a depressive effect of *M arthritidis* on the antibody response to bacteriophages when inoculated simultaneously into rats has been reported (9) and the simultaneously inoculation of membranes of *M arthritidis* and immunogens of *E coli* inhibited the antibody response to *E coli* in rabbits (2) This inhibitory effect may be due to antigenic competition

In the natural situation infections with mycoplasmas and bacteria are not always simultaneous and since this and other studies (12) have shown that where bacterial invasion follows mycoplasmal invasion lymphocyte reactivity and antibody response remain normal then the facilitating effect of mycoplasmas on subsequent bacterial infection would not appear to be due to impairment of these functions

However an inhibitory effect *in vitro* of *M arthritidis* and *M hominis* on the ability of neutrophils to cause phagocytosis of *E coli* was found in this study and by *Simberkoff & Elsbach* (13) Moreover in the present study this effect was shown to occur *in vivo* The intraperitoneal inoculation of 10^8 c f u of *M arthritidis* and *M hominis* impaired the ability of neutrophils in peritoneal exudate to phagocytize *E coli* This would not appear to be an *in vitro* effect caused by mycoplasmas adherent to the leukocytes since in accordance with the findings of *Simberkoff & Elsbach* (13) mycoplasmas could not be cultivated from the sediments of centrifuged leukocyte suspensions

The mechanism by wh h [illegible]

[illegible] employed Thus, the effect seems to be local and dose-dependent which may indicate the possible nessecity of direct contact between the neutrophils and a certain quantity of mycoplasmas This is on a par with the hypothesis that mycoplasmas may damage the cell surface of the neutrophils (13) These observations offer a possible explanation for the facilitating effect of some mycoplasmas on subsequent bacterial infections

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Acta path microbiol scand Sect. C 87 73–77 1979

INTERACTION OF THE Fc PART OF IgG WITH LANCEFIELD EXTRACTS OF HEMOLYTIC STREPTOCOCCI

Strain Specificity and Activity

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Christensen P Burova L A Grubb A Grubb R Samuelsson G Schalen C & Svensson M L. Interaction of the Fc part of IgG with Lancefield extracts of hemolytic streptococci Strain specificity and activity Acta path microbiol scand Sect C 87 73–77 1979

Lancefield extracts of 19 types of group A streptococci as well as one group C and one group G strain were examined for agglutination of human red cells coated with various anti Rh antibodies Fourteen extracts agglutinated one or more of the coated cell samples while five did not The agglutination was inhibited by Fc but not by Fab fragments of human IgG After mouse passages three of the non agglutinating strains acquired agglutinating capacity At least three different reactivities were distinguished by the action of the extracts on IgG1 and IgG3 coated cells respectively Two of the streptococcal extracts agglutinating the same anti Rh coated cells could be further differentiated in hemagglutination inhibition (HAI) experiments using purified IgG3 myeloma proteins Five selected agglutinating systems were inhibited by purified myeloma proteins of the IgG1 IgG2 and IgG4 subclasses IgG3 proteins inhibited only two of the five HAI systems

Key words Streptococci Lancefield extract iso-allotypes Gm markers human Ig Fc fragment.

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Accepted as submitted 11 x 78

The capacity of Lancefield extracts of group A streptococci to agglutinate sheep red cells coated with rabbit anti-sheep red cell antibodies (SRBC) was demonstrated in 1974 (4) This reactivity is widespread among group A streptococci (8) Recently Lancefield extract of group A type 15 streptococci was found capable to agglutinate Rh positive cells coated with »incomplete« anti Rh antibodies and to form precipitate in gel with human sera The reactivity of this extract was restricted as determined with sera from various populations and its capacity to agglutinate red cells coated with various anti Rhs differed (15) In the present paper extracts from different streptococcal groups and types were investigated for restriction in affinity for human Ig

MATERIALS AND METHODS

Streptococcal Strains

The following reference strains of group A streptococci were used M3 (BG 930/24) M5 (100065) M6 (8302) M8 (8324) M9 (100067) M11 (100068) M12 (R53) M15 (100070) M17 (8304) M22 (EF 1950) M27 (EF 1913) M46 (8230) M55 (100189) M56 (100191) and M57 (100190) Furthermore the following *Griffith* strains were used T13 (Glover) and T44 (Henson glossy) 81C and 113G belonging to group C

and G, respectively, have previously been used (5). Group A, T type 28, opacity factor positive (OF+) streptococci were isolated during an epidemic outbreak of streptococcal sore throat in a day nursery. T-typing and test for OF were performed as described (1, 11). 47 of 90 children were infected with the strain.

Mouse Passage of Streptococcal Strains

The strains M3 (BG 930/24), M12 (R53) and M46 (8230) were subjected to mouse passages over several years. Each passage was performed by intraperitoneal injection of mouse spleen suspension taken from dead mice within 24 hours.

Streptococcal Extracts

Group A streptococci, types M15, T28, T44 and M55, were cultured in 20 litres and the other strains in 0.3 litres of Todd-Hewitt broth, cultivation and extraction *ad modum Lancefield* were performed as described elsewhere (15). Several extracts of each strain were prepared and used in repeated experiments.

Human Sera

Sera were obtained from the staff at the above-mentioned day nursery and from the parents of the children. The sera were heated at 56°C for 30 min before use.

M-Components

The M-components were purified from plasma samples from patients with multiple myeloma. A combination of ammonium sulphate precipitation, ion-exchange and gel chromatography and preparative agarose gel electrophoresis (10) was used to isolate the M-components. Their purity was shown by analytical agarose gel electrophoresis (10), immunoelectrophoresis (7) and SDS polyacrylamide electrophoresis (16). Contaminating proteins were below 2% in the preparations. The M components were dialysed against distilled water, lyophilized and stored at room temperature. Stock solutions containing 2 mg protein/ml 0.9% NaCl, determined as described (13) were prepared for the experiments.

Determination of the Subclass of the IgG M-Components

The chemical typing procedure of *Frangione et al.* (6) was used.

Fab- and Fc-Fragments of Polyclonal IgG

Polyclonal IgG was prepared from a pool of plasma from several hundred registered blood donors by gel chromatography on DEAE-Sephadex A 50 (Pharmacia Fine Chemicals, Uppsala, Sweden) equilibrated in 0.05 mol/l Tris-buffer, pH 7.4. Appropriate fractions were pooled and further purified by gel chromatography on Sephadex G-200 (Pharmacia Fine Chemicals). Fab and Fc fragments of the polyclonal IgG were prepared by mercury-papain digestion essentially as described by *Hsiao & Putnam* (9) except that the digestion was terminated after 30 min by addition of recrystallized iodoacetic acid. The fragments were isolated from undigested IgG by gel chromatography on Ultrogel AcA54 (LKB, Stockholm, Sweden) and the Fab and Fc fragments separated by preparative agarose gel electrophoresis (10). The preparations of the fragments were desalted and lyophilized.

Agglutination and Agglutination Inhibition Tests

These tests were performed essentially as described previously (15). The anti-Rh Ripley (Ri) was a gift from Dr. *Marion Waller*, Richmond, USA; this anti Rh is exceptional as it is complement-fixing. Anti Rh KM and 317 are useful for detection of the genetic markers G1m(1) and G1m(4), respectively. The anti Rh HUN detects IgG3 markers which are G3m(5) and is as high titred an anti-Rh as Ri, on analysis with conventional techniques. For coating, the anti-Rhs were diluted as indicated in Table I.

Demonstration of Opacity Factor (OF) and Anti OF Antibodies

OF from group A streptococci type T28 was demonstrated as described by *Maxted et al.* (14). The titre of OF-antibodies was expressed as the highest dilution of serum inhibiting the opacity reaction.

RESULTS

Agglutination of Red Cells Coated with Incomplete Anti-Rh by Lancefield Extracts

The hemagglutination titres of the extracts for red cells coated with four samples of high titred anti-Rh antibodies are given in Table I. Of the 19 extracts, 14 agglutinated one or more of the coated cell samples. Types M11 and M17 streptococci are excluded, as Lancefield extracts from these two strains weakly agglutinated uncoated red cells. It is apparent that the titres for cells coated with Ri are much higher than for cells with other anti Rhs. Seven of the extracts, including those of the group C and G strains, still agglutinated the Ri-coated cells on dilution more than 1000 times. All extracts with a »Ri-titre« of 640 or more agglutinated the cells coated with at least two other anti-Rhs. The results with the IgG1 coats KM and 317 were very similar. It was notable that the M56 extract was active for the IgG1 coats but was inactive for the IgG3 coat (HUN). This was in contrast to M8, M15, group C and group G, which were active for both, and T44, which was active for IgG3 alone.

Acquisition of Ig Binding Activity by Mouse Passage of Strains

The extracts of the three group A streptococcal strains M3, M12 and M46 marked with »x« in Table I did not agglutinate any of the coated cell preparations. After repeated mouse passage of the

ABLE 1 *Agglutination of 0 Rh+ Red Cells Coated with Human IgG Anti Rh by Lancefield Extracts of Streptococci*[a]

xtract of treptococcal roup Strain		Agglutination titre of extracts for red cells coated by anti Rh			
		R_1 (1 15)[b]	KM (1 5)[b]	317 (1 5)[b]	HUN (1 10)[b]
	M3[c]	<10	0	0	0
	M4	1280	4	4	0
	M5	40	0	0	0
	M6	<10	0	0	0
	M8	20000	8	16	8
	M9	20	0	0	0
A	M12[c]	<10	0	0	0
	T 13	20	0	0	0
	M15	20000	16	16	16
	M22	40	0	0	0
	M27	<10	0	0	0
	T 28	640	4	8	0
	T 44	20	0	0	4
	M46[c]	<10	0	0	0
	M55	20000	32	16	4
	M56	2560	16	16	0
	M57	<10	0	0	0
C	81C	2560	32	8	16
G	113G	2560	128	32	64

[a] The extracts of M11 and M17 are excluded from the table as agglutination of uncoated cells occurred

[b] Indicates the dilution of the anti Rh at coating

[c] Before being subjected to mouse passages See text

strains the extracts agglutinated R_1 (diluted 1 15 at coating) in a dilution of 1 180–360 and HUN (1 10) in a dilution of 1 16 KM (1 5) or 317 (1 5) coated cells were not agglutinated

Myeloma Proteins as Inhibitors of Hemagglutination Systems

As mentioned above, 14 of the 19 samples agglutinated one or more of the four coated cell

TABLE 2 *Hemagglutination Inhibition by Purified Myeloma Proteins*

HAI systems	Number of myeloma proteins inhibitory[a]					
	IgG1	IgG2	IgG3	IgG4	IgD	IgM
M15/KM	5	2	0	3	0	0
M15/HUN	5	2	4	3	0	0
T28/R_1	5	2	0	2	0	0
T44/R_1	5	2	0	3	0	0
113G/KM	5	2	4	3	0	0
Total number of myeloma proteins tested	5	2	4	3	1	3
Total number of HAI systems inhibited	5/5	5/5	2/5	variable	0/5	0/5

[a] Inhibitory at a concentration of 0 1–0 01 mg/ml Myeloma proteins not inhibitory at 2 mg/ml were registered as non inhibitory

samples In all the results recorded in Table 1 make 36 hemagglutination systems available for inhibition studies The 18 purified myeloma proteins at our disposition have so far been tested for their capacity as inhibitors in 5 systems representing different streptococcal agglutinating activities M15/KM (streptococcal extract/anti Rh coat) M15/HUN T28/R1 T44/R1 and 113G/KM (Table 2) The five IgG1 and the two IgG2 myeloma proteins were inhibitory at 0 1–0 01 mg/ml in all five HAI systems The IgG3 myeloma proteins were inhibitory only in two namely M15/HUN and 113G/KM The IgG4 myeloma proteins were inhibitory in all systems with the exception of one myeloma protein which was less inhibitory in the T28/R1 system None of the HAI systems was inhibited by the IgD or the three IgM myeloma proteins (2 mg/ml)

Fab and Fc Fragments of Human Polyclonal Ig as Inhibitors

Fc fragments were inhibitory at 0 01–0 001 mg/ml in all of the five HAI systems tested No inhibition was obtained by Fab fragments at 0 5 mg/ml

Lack of Correlation between Anti OF Antibody Titre of Sera and Inhibitory Titre of Sera in HAI System T28/R1

Sixty sera sampled from parents and staff engaged in a type T28 SOR + epidemic outbreak in a day nursery were investigated for inhibitory capacity in the HAI system T28/R1 The anti OF T28 antibody titres ranged from 1 1 to 1 640 in 21 sera while 39 did not contain such antibodies Absence or presence of anti OF T28 antibodies did not correlate to the inhibitory capacity of the sera neither did the titres of OF antibodies

DISCUSSION

It is apparent from Table 1 that *Lancefield* extracts from streptococcal strains vary markedly in their capacity to agglutinate red cells coated with anti Rh The titres of the extracts for the R1 coated cells were considerably higher than for the cells coated with the other anti Rhs It should be noted that the R1 coat is exceptional among anti Rhs since it detects rheumatoid factors much more effectively than do other coats The reasons for this behaviour of R1 are not well understood Extracts from strains M8 M15 and M55 could be diluted more than 20 000 times and still agglutinate the red cells coated with R1 whereas the undiluted extracts of M3 M6 M12 M27 and M46 were inactive All strains except T28 81C and 113G were reference strains and have thus been recultivated in the laboratory for several years

The extracts of strains M3 M12 and M46 agglutinated both R1-coated and HUN-coated cells after mouse passage in contrast to the extracts obtained before the passages which were inactive These strains were M negative after the prolonged passages yet highly virulent for mice as compared with the original strains (2) Studies are in progress to elucidate whether the agglutinating capacity can also be increased in this way in strains showing low activity or induced in other negative strains *Havlicek* (8) found that all of 38 fresh isolates from an epidemic outbreak agglutinated SRBC while only 38 of 175 collection strains did so This might indicate that the agglutinating capacity could be lost during storage of the strains However it remains to be clarified whether some strains of certain types might lack capacity to agglutinate anti Rh coated cells also when freshly isolated from clinical sources or after mouse passages *Havlicek* (8) found no activity for SRBC amond 28 type 12 group A streptococci 13 of which were collection strains and 15 freeze dried cultures On the other hand our results might indicate that the capacity to agglutinate sensitized red blood cells is inducible in type 12 and lack of this characteristic thus may not be a constant property of this particular type

Only IgG proteins were inhibitory in the five HAI systems tested Neither the IgD nor three IgM myeloma proteins were inhibitory in any of the systems IgA proteins have so far not been studied As inhibition was obtained by Fc fragments of IgG but not by Fab fragments it is reasonable to assume that the anti Ig activity in the streptococcal extracts is identic with the IgG Fc receptor of streptococci (3) Furthermore *Havlicek* (8) found that the Fc part of rabbit IgG inhibited streptococcal agglutination of SRBC

The Lancefield extracts of M15 T44 and M56 streptococci gave different agglutination patterns with red cells coated with KM and HUN anti Rhs useful for detection of IgG1 and IgG3 genetic markers respectively To further elucidate the restrictions in reactivity for human immunoglobulins expressed for the different extracts the inhibitory effect of myeloma proteins on the hemagglutination was studied The reactivity of the extracts in HAI experiments varied from one streptococcal type to another We therefore considered the possibility that inhibition by a myeloma protein in one system but not another could be due to antistreptococcal type specific antibodies Test of a number of human sera with and without type specific antibodies for the T28 strain in the T28/R1 HAI system showed no correlation between inhibition and presence of

type specific antibodies However it is necessary to further elucidate the possible influence of antibodies directed against other streptococcal components before the outcome of HAI studies with normal sera can be attributed to inhibition only by the Fc parts

The M15 and 113G extracts were similar in the sense that they agglutinated both KM and HUN sensitized cells However in HAI systems involving the KM coat and IgG3 myeloma proteins these extracts behaved differently (Table 2) Including the finding that some extracts were non reactive with anti Rh coated cells five different affinities for human IgGs could be registered exemplified by M3 (non reactive) M4 (reactive with IgG1 coats only) –

M15 a

subclas

restricted activity of the M15 extract was further illustrated by the finding that the four IgG3 myeloma proteins inhibited the agglutination of the IgG3 coated cells but *not of the IgG1 coated cells* (Table 2)

The five IgG1 proteins belonging to G1m(1) or *G1m(4) all inhibited the five HAI* systems investigated indicating that the inhibition was caused by a marker that is isotype for the IgG1 subclass The observations of *Kronvall* (12) indicated differences between two IgG3 myeloma sera in affinity for a group A streptococcal strain All the IgG3 myeloma *proteins* used in the present study *were Gm(5)* which renders it less likely that these proteins should differ with respect to genetic markers *in the* Fc part It was also found that all *four IgG3* myeloma proteins varied in the same way in the different HAI systems *That differences* within one IgG subclass could *be de ected in HAI* systems involving *streptococcal* extracts was elucidated by the *d ffering* behaviour *of one of the three IgG4* myeloma proteins in the T28/Rt system The similarity in the inhibition patterns of the IgG1 myeloma proteins combined with the *results* obtained with the IgG4 myeloma proteins may indicate that some of the extracts have affinity *for* *iso-allotypic* markers as our earlier experiments have suggested (15)

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BRIEF REPORT

QUANTITATION OF $C\bar{1}r$ $C\bar{1}s$ $C\bar{1}$ INACTIVATOR COMPLEXES BY ELECTROIMMUNOASSAY

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Laurell A B Mårtensson, U & Sjoholm A G Quantitation of $C\bar{1}r$-$C\bar{1}s$ $C\bar{1}$ inactivator complexes by electroimmunoassay Acta path microbiol scand Sect. C 87 79–81 1979

A method for measuring complexes of $C\bar{1}r$-$C\bar{1}s$-$C\bar{1}$ IA in normal and pathological human sera was developed The electroimmunoassay was applied and benefit was derived from the antigenic and electrophoretic properties of the known C1 subcomponent complexes in serum The concentrations of $C\bar{1}r$-$C\bar{1}s$-$C\bar{1}$ IA complexes of 20 normal sera were determined Storage of serum at room temperature markedly increased the values over those obtained from fresh specimens while freezing and thawing did not influence the results

Key words C1 subcomponent complexes C1 activation Electroimmunoassay

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C1 subcomponent complexes composed of $C\bar{1}r$ $C\bar{1}s$ and $C\bar{1}$ inactivator ($C\bar{1}r$-$C\bar{1}s$-$C\bar{1}$ IA complexes) or of [illegible] complexes containing C reactive protein and with proteolytic enzymes such as $C\bar{1}r$ $C\bar{1}s$ and trypsin It was concluded that $C\bar{1}r$ $C\bar{1}s$-$C\bar{1}$ IA complexes in serum signify activation of C1 (5 8)

The present study concerns the measurement of $C\bar{1}r$ $C\bar{1}s$-$C\bar{1}$ IA complexes in human serum by electroimmunoassay

Materials and Methods

Serum samples Blood specimens were obtained from 20 apparently healthy individuals and from patients with rheumatoid arthritis chronic urticaria relapsing pneumococcal otitis media or hereditary angio-edema (HANE) The sera were frozen in aliquots at −80° C within 4 hours after sampling

Heat aggregated IgG Commercial IgG (AB Kabi Stockholm Sweden) at 10 mg per ml pH 6 5 was heated at 63° C for 30 minutes

Reference serum Pooled normal serum was incubated with heat aggregated IgG at 1 mg per ml for 60 minutes at 37° C After incubation all C1s antigen was present in the $C\bar{1}r$-$C\bar{1}s$-$C\bar{1}$ IA complex as detected by crossed immunoelectrophoresis (8) The serum treated with heat aggregated IgG was used as a reference for determinations of $C\bar{1}r$ $C\bar{1}s$-$C\bar{1}$ IA complexes by electroimmunoassay

Partially purified $C\bar{1}r$ $C\bar{1}s$ $C\bar{1}$ IA complexes Euglobulin obtained by precipitation at pH 5 5 (3) was fractionated on DEAE cellulose in phosphate buffer pH 7 4 Fractions containing $C\bar{1}r$ $C\bar{1}s$-$C\bar{1}$ IA complexes as recognized by electroimmunoassay (8) were pooled and concentrated Further purification was obtained by gel filtration on Sephadex G200 Concentration of fractions was carried out by pressure dialysis (XM 50 Amicon B V Oosterhout/N B / Holland)

Dilutions of the preparation were used in the electroimmunoassay for construction of a reference curve covering the desired range of $C\bar{1}r$-$C\bar{1}s$-$C\bar{1}$ IA concentrations

Monospecific antisera Rabbit antisera to C1r C1s and $C\bar{1}$ IA were obtained as described previously (5 10)

Quantitation of $C\bar{1}r$ $C\bar{1}s$ $C\bar{1}$ IA complexes

The electroimmunoassay (9) was employed using 0 075 M barbital buffer pH 8 6 with Ca^{++} at 2 mM

per l The agarose gel was cast in two steps The cathodal gel section contained anti C1r and the anodal gel a mixture of anti C1r and anti C1s Holes were punched into the cathodal gel Undiluted normal and pathological sera in 5 μl volumes were applied as also the reference serum pool diluted ½, and appropriate dilutions of partially purified $C\bar{1}r$ $C\bar{1}s$ $C\bar{1}$ IA preparation (Fig 1) Electrophoresis was carried out at 4 V per cm for 20 hours Values were given as percentages of the $C\bar{1}r$ $C\bar{1}s$ $C\bar{1}$ IA concentration in the reference serum which was said to be 100 per cent

Under the conditions used macromolecular C1 (C1qrs) remained at the application site and C1r C1s complexes were precipitated in the cathodal gel section containing anti C1r On the other hand $C\bar{1}r$ $C\bar{1}s$ $C\bar{1}$ IA complexes were not retained in the cathodal gel but formed sharply out lined precipitates in the anodal gel section containing anti C1r and anti C1s (Fig 1)

Results

$C\bar{1}r$ $C\bar{1}s$ $C\bar{1}$ IA complexes in normal sera The observed range of $C\bar{1}r$ $C\bar{1}s$ $C\bar{1}$ IA concentrations in 20 freshly thawed sera was 11–25 per cent with a mean value of 16 per cent On double determinations the error of the method was found to be 1 4 per cent (SD)

Normal sera were tested after storage at room temperature for 1 2 3 and 5 days and after freezing and thawing 3 times After storage at room temperature for 1 day $C\bar{1}r$ $C\bar{1}s$ $C\bar{1}$ IA values increased to about the double of those found in freshly thawed sera On longer storage increasing values were noted up to about 50 per cent of the reference serum Freezing and thawing up to 3 times did not influence the $C\bar{1}r$ $C\bar{1}s$ $C\bar{1}$ IA values

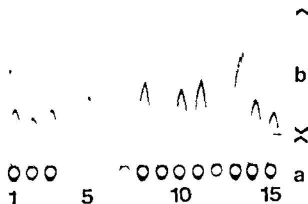


Fig 1 Electroimmunoassay of $C\bar{1}r$ $C\bar{1}s$ $C\bar{1}$ IA complexes in normal and pathological sera Holes 1–3 normal sera 4–6 purified preparation of $C\bar{1}r$ $C\bar{1}s$ $C\bar{1}$ IA undiluted ½ and ¼ reference serum diluted ½ 8–15 pathological sera 8–9 rheumatoid arthritis 10–11 relapsing acute pneumococcal otitis media 12–13 chronic urticaria 14–15 HANE on treatment with Danazol (14) before treatment (15) Sera were analysed undiluted

a anti C1r in the gel

b anti C1r and anti C1s in the gel

$C\bar{1}r$ $C\bar{1}s$-$C\bar{1}$ IA complexes in pathological sera Sera previously known to contain increased amounts of $C\bar{1}r$ $C\bar{1}s$ $C\bar{1}$ IA complexes as estimated by crossed immunoelectrophoresis were selected for the study In accordance with the previous semiquantitative estimation (4 6) sera from patients with rheumatoid arthritis chronic urticaria relapsing pneumococcal otitis media or hereditary angio edema gave high $C\bar{1}r$ $C\bar{1}s$ $C\bar{1}$ IA values in the electroimmunoassay In some cases values about 50 per cent of the reference serum were obtained (Fig 1) In one patient with HANE samples obtained before and after treatment with Danazol were compared This drug is known to increase the serum levels of C1 IA in HANE (2) Before treatment the $C\bar{1}r$ $C\bar{1}s$ $C\bar{1}$ IA value was 19 per cent and after treatment 24 per cent of the reference serum (Fig 1)

Discussion

Three species of C1 subcomponent complexes can be identified in human sera by crossed immunoelectrophoresis 1 the classical C1 complex of C1q C1r and C1s which is active in immune hemolysis 2 a complex of proenzyme C1r and C1s and 3 the $C\bar{1}r$ $C\bar{1}s$ $C\bar{1}$ IA complex in which activated C1r and C1s molecules have reacted with the $C\bar{1}$ IA (4 5 6 8) Differences in electrophoretic and antigenic properties of these complexes were utilized in the electroimmunoassay to allow quantitative analysis of $C\bar{1}r$ $C\bar{1}s$ $C\bar{1}$ IA complexes in serum On agarose electrophoresis in the presence of Ca^{++} C1r-C1s and $C\bar{1}r$ $C\bar{1}s$ $C\bar{1}$ IA complexes migrate towards the anode whereas the classical C1 complex does not enter the gel In the electroimmunoassay C1r C1s complexes were precipitated in a cathodal gel section by anti C1r $C\bar{1}r$ $C\bar{1}s$ $C\bar{1}$ IA complexes did not precipitate with anti C1r alone which appears to be related to the previously described antigenic silence of $C\bar{1}r$ (5 8 12)

The combination of anti-C1s with anti C1r in the anodal gel section was found to be necessary in order to obtain well defined precipitates This is in accordance with earlier studies by crossed immunoelectrophoresis in which anti C1s alone gave a diffuse precipitate with the $C\bar{1}r$ $C\bar{1}s$ $C\bar{1}$ IA complex and the addition of anti C1r resulted in a sharply out lined precipitate when the second electrophoretic step was performed in the presence of Ca^{++} (5 8)

Combination of anti C1s with anti $C\bar{1}$ IA in the anodal gel was not suitable for the estimation of $C\bar{1}r$ $C\bar{1}s$ $C\bar{1}$ IA complexes due to the excess of native $C\bar{1}$ IA present in serum

Single radial immunodiffusion was used for determination of free C1s in serum (12 13) However this method will hardly discriminate between C1r C1s and $C\bar{1}r$ $C\bar{1}s$ $C\bar{1}$ IA complexes

The investigation showed a considerable increase of $C\bar{1}r$ $C\bar{1}s$ $C\bar{1}$ IA values in serum already after one day of storage at room temperature Thus determinations of $C\bar{1}r$ $C\bar{1}s$ $C\bar{1}$ IA complexes must be performed in fresh sera or in sera frozen at −80° C after sampling

The method described may be useful for *in vitro* studies of C1 activation and for the assessment of C1 activation in disease As will be reported elsewhere

studies of serial samples from patients with rheumatoid arthritis and acute post streptococcal glomerulonephritis indicate that the concentrations of C$\bar{1}$r C$\bar{1}$s C$\bar{1}$ IA complexes correlate with disease activity and decrease on clinical improvement (1 11)

It is of interest that treatment with Danazol in a patient with HANE gave increased amounts of C$\bar{1}$r-C1s C$\bar{1}$ IA complexes in serum Further stud es are required to evaluate this finding

This work was supported by grants from the *Swedish Medical Research Council* (B 78 16 00068) *Riksförbundet mot Reumatism Greta och Johan Kocks Stiftelser* and *Foreningen for Vanföra I Skåne*

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IN VITRO EFFECT OF R 17934, A NEW DRUG WITH ANTITUBULIN ACTIVITY, ON NEUTROPHIL GRANULOCYTE LOCOMOTION AND ORIENTATION

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Valerius N H In vitro effect of R 17934 a new drug with antitubulin activity on neutrophil granulocyte locomotion and orientation Acta path microbiol scand Sect C, *87* 83-89 1979

R 17934 is a new synthetic antimicrotubule drug which binds to tubulin at the same site as does *colchicine The interaction of* this drug with human neutrophil granulocyte locomotion and orientation *was examined using* a modified reversible Boyden chamber and a gradient chamber offering a direct visual assay of cell orientation R 17934 at concentrations as low as 5×10^{-6} M was *found to* inhibit chemokinesis to an equal *degree in gradient chambers* and in Boyden chambers using checkerboard experiments in wh...

agent were varie...

chambers Much ...

neutrophils to o... in gradient chambers This finding may show that filters form a more suitable substrate for *oriented pseudopod formation* of microtubule-deficient cells than do glass or plastic slides In conclusion this study has provided *evidence that in the absence of* ...

...lating a recognition signal into locomotion The previous finding that colchicine *also had this* effect supports the hypothesis that this process may be microtubule-dependent

Key words chemotaxis leukocyte locomotion microtubule neutrophil R 17934

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Much evidence now points towards a role of cytoplasmic microtubules in the *regulation of* leukocyte locomotion (2) This evidence was primarily derived from the findings of ...

that microtubules re-orient themselves within cells exposed to reversing gradients of cytotaxins (10) have supported the hypothesis that microtubules are important for the ability of leukocytes to *migrate* directionally towards the source of a concentration gradient of chemotactic factors i e chemotaxis However, the demonstration that cytotaxins *also induce* an increased nondirectional migration of leukocytes *in the* absense of a concentration gradient i e chemokinesis (9 *16, 19)* has made it pertinent to include a distinction *between* chemo-

taxis and chemokinesis when examining leukocyte locomotion This distinction can be made using the Boyden technique in checkerboard assays (17 19) in which the concentration of the chemoattractant is varied above and below the filters in a series of tests Indeed, it has been shown in such a study (15) that colchicine inhibited chemokinesis rather than chemotaxis

Oncodazole (R 17934 Methyl (5 (2-thienylcarbonyl)-1H benzimadazol 2 yl) carbamate Janssen Pharma) is a new microtubule inhibitor (6) which interferes with the binding site of colchicine on *tubulin* and which has been shown to affect microtubule function in human polymorphonuclear leukocytes (PMN) (11)

The purpose of the present study is to further delineate the possible role of microtubules in leukocyte locomotion and orientation by examining the effect of R 17934 on these functions PMN chemotaxis chemokinesis and spontaneous motility were determined using a modified reversible Boyden chamber in checkerboard assays The ability of PMN to orient was examined with a technique recently introduced (18) which allows phase microscopic observations of cells exposed to controlled gradients of chemotactic factors

MATERIALS AND METHODS

Chemotaxis and Chemokinesis Assays

The PMN chemotactic activity was determined in a modified reversible Boyden chamber as previously described (14) Briefly leukocytes from healthy donors were suspended in Gey s balanced salt solution (GBSS) Red cells were removed by dextran sedimentation and hypotonic lysis and the concentration of PMN corrected to 1 × 10^6 per ml The chemotactic agent used was a culture filtrate of *E coli* (BCF) diluted 1 3 with GBSS if not otherwise stated In the upper compartment of the Boyden chamber 5 × 10^5 PMN were placed separated from the chemotactic agent by a filter with a pore size of 3 microns (Sartorius Gottingen Germany) The chambers were incubated for 2 ½ h at 37° C

After 2 h of the incubation period the chambers were turned upside down to prevent detachment of the cells from the lower surface of the filter The filters were fixed and stained with haematoxyline and then dehydrated and cleared in xylene The cells which had migrated completely through the filters and were lying on the attractant surfaces were counted using an automatic image analysis system (Classimat Leitz Wetzlar Germany) The chemotactic activity was expressed as the mean number of cells per screen field at 10 × 10 magnification All experiments were performed in triplicate and the median value used in reporting the results

The distinction between *chemotaxis* and *chemokinesis* was studied using the checkerboard assay first described by *Zigmond & Hirsch* (19) In these experiments a series of chambers was used in which the varying concentrations of the chemoattractant were placed either above the filter below the filter or in both compartments Each set of triplicates thus differed from all others with respect to the absolute concentration of the attractant the concentration gradient or both Details of the concentrations used appear in Table I

Cell suspensions containing 2 × 10^6 PMN per ml GBSS without albumin were incubated with R 17934 (generously supplied by Janssen Pharma Copenhagen Denmark) at varying concentrations as indicated for 60 min at 37° C After this incubation the cell suspensions were diluted with equal volumes of GBSS containing 4 % human albumin before being tested for leukotactic activity in the continued presence of R 17934 at half the initial concentration In the experiments using cells pre incubated with either BCF diluted 1 3 with GBSS or with GBSS pre incubation was performed for 30 min at 37° C After this the cells were washed twice in GBSS before incubation with R 17934 All experiments included control samples incubated without R 17934

Orientation Assays

The gradient chamber was made as described by *Zigmond* (18) (Molytex Rodovre Denmark) Two parallel grooves 1 mm deep and 4 mm wide were cut 1 mm apart across an acryl plastic slide 26 mm × 76 mm × 3 mm A 24 mm × 32 mm glass cover slip was held tightly down to the acryl plastic slide by two small bull dog clamps (Myers & Sons Ltd Oldbury Birmingham England) Before use the cover slips and the acryl plastic slides were carefully cleaned with ethanol 70 % and air dried The gradient chambers were assembled by inverting a cover slip with cells attached across its center region onto the plexiglass slide allowing the cells to lie on the bridge between the two grooves After fixing the cover slip to the plast slide with the bull dog clamps one groove was filled with GBSS containing 1% gelatine (Merck Darmstadt Germany) The opposite well was

(N formyl

Co St

10^{-6} M in

GBSS containing 1% gelatine The chambers were incubated for 30 min in a moist chamber at room temperature before reading

Cells Pure (more than 99 %) PMN suspensions were prepared from heparinized venous blood from 8 healthy donors by centrifugation on Lymphoprep (Sodium metrizoate Ficoll Nyegaard & Co Oslo Norway) followed by dextran sedimentation of the red cells as described by *Bøyum* (4) The remaining erythrocytes were removed by hypotonic lysis The PMN were suspended in GBSS at a concentration of 4 × 10^5 PMN per ml Incubations with various concentrations of R 17934 were performed as described above

Scoring of cell polarization and orientation The cells lying on the bridge were observed with a 10 × 40 phase contrast microscope Polarization of the cells was indicated by the presence of a broad anterior lamellipodium and a posterior knob like tail The cells were scored as oriented either towards or away from or perpendicu

lar to the concentration gradient of the chemotactic agent In each chamber at least 100 polarized cells were scored The number of cells which showed no polarization after the incubation period was also determined The results were expressed as the number of cells oriented towards the source of the gradient × 100 divided by the total number of polarized cells The number of not polarized cells was expressed as a percentage of the total number of cells counted All experiments were done in duplicate and the results represent the mean The reading was done blindly

Viability

This was assured by the trypan blue exclusion test

Statistics

Mann Whitney rank sum test, Friedman test

RESULTS

It will be seen from Fig 1 that preincubation of PMN with varying concentrations of R 17934 results in a dose-dependent inhibition of their leukotactic response This inhibition was significant at concentrations down to 5×10^{-6} M At lower concentrations of R 17934 there was no significant difference between the activity of cells preincubated with R 17934 and control cells It can also be seen

curves are nearly identical

The effect of R 17934 on chemotaxis and chemokinesis induced by BCF can be seen from Table 1 In these experiments the cells were allowed to migrate through the filter from lower to higher concentrations of BCF (positive concentration gradients) and from higher to lower concentrations of BCF (negative gradients) Cells were also allowed to migrate in the absence of a concentration gradient i e with equal concentrations of BCF on both sides of the filter The chemokinetic activity (the activity observed in the absence of a concentration gradient) increased for both R 17934 treated cells and untreated cells as the concentration of BCF was increased However this increase was much lower for cells treated ...

... therefore inhibited chemokinesis at the higher concentrations

The concentration gradient across the filter was positive above the diagonals from upper left to lower right in the tables and negative below Thus directional migration towards the source of the gradient is indicated by higher figures above than below these diagonals The influence of the concentration gradient at various absolute concentrations of BCF is seen along the lines parallel to the diagonals from lower left to upper right Along these lines the concentration gradient reversed from being negative to positive while the absolute concentration of BCF was kept constant In all experiments using untreated cells the leukotactic activity increased along these lines indicating a chemotactic activity The results of incubation with R 17934 at a concentration of 10^{-6} M (Table 1 C) were not different from those of the simultaneous control experiments While the activity of cells incubated with R 17934 at a concentration of 5×10^{-6} M (Table 1 B) was generally lower than that of the control cells as a result of inhibited chemokinesis, the proportional increase in the activity along the lines from lo...

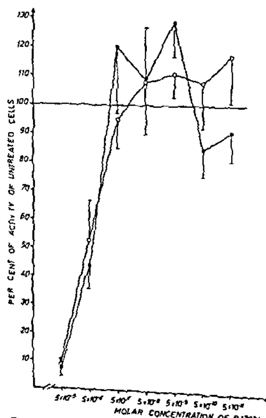


Fig 1 Effect of R 17934 on the leukotactic activity of PMN pre incubated with either BCF (diluted 1 3) (closed circles) or Gey s solution (open circles) Abscissa Molar concentration of R 17934 Ordinate Leukotactic activity in percent of the activity of non treated PMN Mean ± 1 SEM of 4 experiments

TABLE 1 *Effect of Varying the Concentration Gradient and the Absolute Concentration of a Bacterial Chemotac Factor (BCF) on the Locomotory Response of Human Neutrophil Granulocytes Pre incubated with either Gey s Solut (GBSS) or R 17934 at Varying Concentrations for 60 Min at 37° C The Results are the Means of Two Experime*

			Concentration of BCF (%) in Attractant Compartment					Concentration of BCF (%) in Attractant Compartment				
			0	12	20	32		0	12	20	32	
A	Concentration of BCF (%) in Cell Compartment	0	7	8	24	29	R 17934 10 5 M	12	22	45	112	GBSS
		12	17	19	19	23		10	19	39	93	
		20	10	15	15	23		12	36	69	84	
		32	21	15	22	28		12	35	55	75	
B	Concentration of BCF (%) in Cell Compartment	0	5	28	44	88	R 17934 5×10^{-6} M	10	68	97	162	GBSS
		12	7	19	32	81		20	60	90	131	
		20	10	20	39	72		16	38	73	142	
		32	12	21	32	68		14	48	75	105	
C	Concentration of BCF (%) in Cell Compartment	0	10	62	216	274	R 17934 10^{-6} M	13	147	218	278	GBSS
		12	24	85	160	252		25	116	137	227	
		20	23	134	139	264		25	121	175	223	
		32	27	136	179	237		24	91	135	169	

and react with directional locomotion towards a concentration gradient However at the highest concentrations of R 17934 tested (10 5 M Table 1 A) also the chemotactic activity seemed to be inhibited since there was no or only a slight increase in the activity along these lines

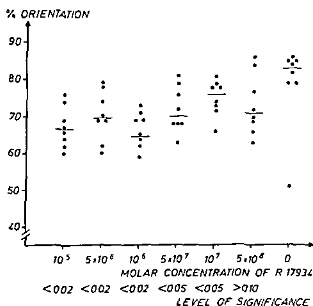


Fig 2 Number of cells oriented towards the source of a concentration gradient of N formyl methionyl phenyl alanine in percent of the total number of polarized cells at various concentrations of R 17934 Results of 8 experiments Statistics Mann Whitney rank sum test

The difference in the activity of the control cells was due to day to day variations possibly as the result of variations of filter quality within the same batch

Fig 2 shows the effect of preincubation with R 17934 on the ability of PMN to orient in a concentration gradient of N formyl methionyl phe nylalanine (NFMP) In the entire concentration range of R 17934 tested (10 5 M – 5 × 10 8 M) more cells were oriented towards the source of the concentration gradient than away from it or perpendicular to it However the percentage of cells which had oriented towards the chemotactic factor decreased with increasing concentrations of R 17934 and was significantly reduced when compa red to untreated cells ($p < 0\ 05$ Mann Whitney rank sum test) at concentrations as low as 10 7 M This effect could be shown to be dose-correlated ($p < 0\ 05$ when the results of untreated cells were excluded and $< 0\ 01$ when they were included Friedman test) This observation shows that leuko- cytes can orient in a chemical gradient when microtubules are disrupted However their level of orientation is significantly decreased

Fig 3 shows the number of not polarized cells as a percentage of the total number of observed cells after incubation with various concentrations of R 17934 The percentage of not polarized cells was significantly higher ($p < 0\ 02$ Mann Whitney rank sum test) in samples incubated in 10 5 M or 5×10^{-6} M R 17934 than in control samples The

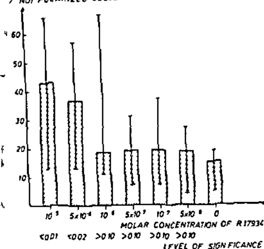


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number of not polarized cells at these concentrations of R 17934 was similar to the number of not polarizing cells found when only buffer solution was added to the wells (not shown here) This indicates that in addition to impairing cell orientation towards a chemotact [illegible]

[illegible]rypan blue exclusion test showed more than 95% dye-excluding cells in all samples after preincubation with various concentrations of R 17934

DISCUSSION

R 17934 is a specific antitubulin which interferes with microtubule function by inhibiting tubulin polymerization into normal microtubules After treatment of various cell lines with R 17934 at concentrations of 10^{-5} M and 5×10^{-6} M cytoplasmic microtubules were completely dissolved within 20 min as judged from electron microscopy (6) R 17934 acts by b[illegible] at the [illegible] bindin[illegible] This [illegible] may account for the finding that the inhibitory action of R 17934 on PMN [illegible] activity [illegible]

activity was examined in the presence of an antitubulin at half the concentrations at which the cells had been preincubated This procedure diminishes the effect of a more reversibly binding agent The effect of R 17934 on leukocyte locomotion has not previously been reported however the finding that this antitubulin inhibits leukotaxis supports numerous previous reports (3 5 12) showing that antitubulins are efficient inhibitors of leukocyte locomotion

The finding that preincubation with BCF does not render the cells more susceptible to R 17934 confirms the result of a similar previous study on the interaction of colchicine with leukocyte locomotion (15) Since exposure of PMN to chemotactic factors has been found to induce microtubule formation (7) it was speculated that the action of antitubulins might be reduced by pre treatment with chemotactic factors The failure to demonstrate this may be the result of the very dynamic state of microtubule assembly and break down which continuously liberates tubulin to be bound by antitubulins (13) Indeed if locomotion itself depends on the equilibrium between tubulin monomers and polymers preincubation with chemotactic factors could not be expected to have any such effect

It has previously been found that demecolcine an active derivative of colchicine inhibited the migration of PMN towards casein in Boyden chambers but affected the velocity of PMN moving randomly on a glass slide to a much lesser extent (3) This observation led to the concept of microtubules as important structures for directional cell locomotion (2) However it was subsequently demonstrated that leukocytes show enhanced non-directional migration when exposed to chemotactic agents not forming a concentration gradient (19) This type of stimulated locomotion has recently been termed chemokinesis (9) as distinct from chemotaxis which is the directional stimulated locomotion towards the source of a concentration gradient Thus a leukotactic response observed using the conventional Boyden technique results from the joint effects of chemokinesis and chemotaxis Inhibition of the activity may therefore be due to inhibition of either or both types of locomotion The checkerboard assay by *Zigmond & Hirsch* (19) is believed to provide the best means available at present to distinguish chemokinesis from chemotaxis (17) The data shown in Table 1 demonstrating that R 17934 has a strong inhibitory effect on chemokinesis are consistent [illegible]

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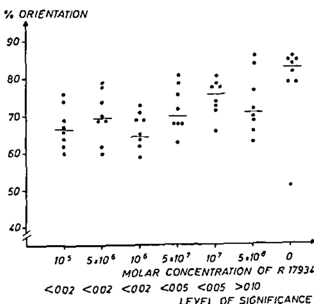


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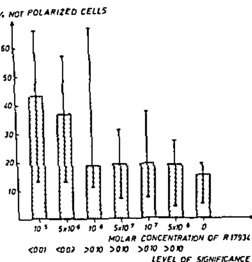


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activity ...
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The finding that preincubation with BCF does not render the cells more susceptible to R 17934 confirms the result of a similar previous study on the interaction of colchicine with leukocyte locomotion (15) Since exposure of PMN to chemotactic factors has been found to induce microtubule formation (7) it was speculated that the action of antitubulins might be reduced by pre-treatment with chemotactic factors The failure to demonstrate this may be the result of the very dynamic state of microtubule assembly and break down, which continuously liberates tubulin to be bound by antitubulins (13) Indeed, if locomotion itself depend ...

effect

It has previously been found that demecolcine an active derivative of colchicine inhibited the migration of PMN towards casein in Boyden chambers but affected the velocity of PMN moving randomly on a glass slide to a much lesser extent (3) This observation led to the concept of microtubules as important structures for directional cell locomotion (2) However, it was subsequently demonstrated that leukocytes show enhanced non-directional migration when exposed to chemotactic agents not forming a concentration gradient (19) This type of stimulated locomotion has recently been termed chemok...

...tactic response observed using the conventional Boyden technique results from the joint effects of chemokinesis and chemotaxis Inhibition of the activity may therefore be due to inhibition of either or both types of locomotion The checkerboard assay by *Zigmond & Hirsch* (19) is believed to provide the best means available at present to distinguish chemokinesis from chemotaxis (17) The ...

at higher concentrations (10^{-5} M) The discrepancy between this finding and the previously reported effect of colchicine is likely to be due to the fact that colchicine was not tested in checkerboard assays at such high concentrations It is possible however that some of the observed phenomena may have been due to non specific effects of higher concentra tions of R 17934 other than on microtubules These high concentrations of R 17934 did not affect PMN viability as judged from the results of trypan blue exclusion test

The introduction of a gradient chamber (18) which offers a visual determination of PMN behaviour in a controlled concentration gradient appears to be an improvement of previously available methods which depend on diffusion of soluble agents from a solid source i e a clump of microorganisms and result in an unstable gradient Using such a chamber colchicine was found not to affect significantly the ability of PMN to orient in response to a concentration gradient of N formyl methionyl leucyl phenylalanine (18) It was not reported whether colchicine affected the number of PMN showing polarization

The results of the present study shown in Fig 2 indicate that while neutrophils can orient towards a concentration gradient after treatment with an anti tubulin such as R 17934 they do so less accurately This effect of R 17934 was significant at concentra tions as low as 10^{-7} M although numerical differences were rather small This finding is consistent with the results of the checkerboard assay shown in Table 1A The fact that this inhibition of directional locomotion could be disclosed at a much lower concentration in the direct visual assay than in the filter assay may be due to the different parameters which they measure The direct visual assay measures cell orientation which requires only enough motility for the cell to polarize in one direction while the filter assay depends on directio nal locomotion of the cells through the filter How ever it has recently been suggested (10) that the formation of pseudopods in colchicine treated neu trophils depends on a suitable surface to which the cells may adhere In the absence of functioning microtubules filter surfaces may offer a substitute exoskeleton onto which cells can adhere and orient Nitrocellulose filters and glass or plast slides provide different conditions for substrate adhesion and may in this way influence the effect of antitubulins on cell locomotion This observation illustrates the need for employing different assay systems when interpreting leukocyte locomotion

- with the
inson (1)
reported

that colchicine treated cells showed directional migration towards spores of *C albicans* but that they showed wider angles of turn than untreated cells so that they moved towards the source of the gradient in more irregular paths

The gradient chamber can assess the proportion of cells showing no polarization unlike other current visual methods which use cinemicrography only to measure the velocity (3) the distance of migration (12) or the angles of turn (1) of the cells Less than 20% of the untreated cells remained un polarized on exposure to a chemotactic gradient Preincubation with R 17934 at concentrations of 10^{-5} M and 5×10^{-6} M significantly increased this number to a median of about 40% This figure is close to that observed when incubating the cells in the gradient chamber in the absence of chemotactic factors i e with buffer solution in both wells This finding suggests that R 17934 at these concentra tions blocks the ability of the cells to respond with locomotion to a chemotactic signal The finding is in close agreement with the results of the checkerboard assays showing that R 17934 at these concentra tions inhibits chemokinesis Thus in contrast to chemotaxis chemokinesis seems to be equally inhibited by R 17934 in glass slide and filter assays

In conclusion this study has provided further evidence that microtubules are involved in cell locomotion by showing that R 17934 a new drug with antitubulin activity inhibits both chemotaxis and chemokinesis The inhibition of chemotaxis was more pronounced in a direct visual assay than in filter assays suggesting that filters offer a more suitable substrate for oriented pseudopod formation than do glass or plastic slides The inhibition of chemokinesis was equal in filter and direct visual assays It is proposed that R 17934 interferes with the mechanism translating a membrane signal into locomotion The previous finding that colchicine also inhibited chemokinesis supports the hypothesis that this process may be microtubule-dependent

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PURIFICATION AND CHARACTERIZATION OF RABBIT ANTI-MOUSE RENIN SPECIFIC Fab FRAGMENTS

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Lykkegård S Purification and characterization of rabbit anti mouse renin specific Fab fragments Acta path microbiol scand Sect. C 87 91-97 1979

Antibodies raised against pure renin from the submaxillary gland of mice were used to obtain renin specific Fab fragments The purification steps were DEAE-chromatography followed by papain digestion with separation of the undigested IgG preparation from the Fab/Fc fragments on a Sephadex G 100 column Finally the Fab fragments were subjected to affinity chromatography on a CH Sepharose 4B column with submaxillary renin attached The purified Fab fragments revealed only a single band in SDS polyacrylamide gel electrophoresis and a single precipitation line in cross immunoelectrophoresis The association constants for the reaction of renin with the purified Fab fragments compared to the divalent antibodies were of the same magnitude $0\,7 \times 10^{11}$ l/mol and $1\,0 \times 10^{11}$ l/mol respectively Comparison of the affinity of the Fab fragments for the antigenic determinants and the enzymatic inhibition of renin were determined to be approximately the same Thus the pure specific immunoreactive Fab fragment of antirenin with an inhibitor constant of $1\,5 \times 10^{-11}$ is the most potent inhibitor of mouse renin so far

Key words Fab fragments renin specific anti mouse antibodies purification characterization

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The periodical interest in antirenin (22 23 and 7) and the possibility of using it to reveal the role of renin in renal hypertension and the physiological role of renin has been more attractive after the purification to homogeneity of renin from the mouse submaxillary gland (2) and the hog kidney (14)

The problems by using antibodies raised against impure renin and the interpretations of the results obtained by the use of such sera have been repeatedly discussed (23)

The aim of the present study was to purify the antibody and especially its active Fab fragments elicited against pure submaxillary mouse renin Subsequently to determine the Fab fragments affinity to renin and its inhibitory effect on the catalytic activity of renin

MATERIALS AND METHODS

Purification of Submaxillary Mouse Renin

The purification of renin from the submaxillary

fractionation followed by 5 steps of column chromatography on Sephadex G 100 diethylamin cellulose carboxymethyl cellulose again diethylaminoethyl cellulose followed by carboxymethyl cellulose A few minor modifications as described by *Malling & Poulsen* (11) were used Renin was measured by antibody trapping of angiotensin I (21)

Antisera

Four rabbits were injected intradermally at multiple spots with 0 25 mg submaxillary renin (pooled fractions I and II (11) in 0 5 ml physiological saline) emulsified in

of an appropriate dilution of rabbit renin specific antibodies or Fab fragments To this mixture was added 1000 µl labelled submaxillary gland renin containing 35 pg 4000 cpm and an appropriate small amount of rabbit plasma used as carrier IgG The mixture was incubated at 4°C for 96 h At the end of the incubation 100 µl of an appropriate dilution of hog anti rabbit gammaglobulin (light and heavy chains) DAKO was added and the incubation was continued at 4°C for additional 48 h Two ml of 50 mM phosphate buffer pH 7 4 were added to the tubes followed by centrifugation at 3000 × g for 30 min The supernatant except for 200 µl containing the precipitate was removed by suction The precipitate was further washed twice followed by centrifugation and removal of the supernatant Finally the radioactivity in the precipitate was counted

Immunoelectrophoresis

Immunoelectrophoresis was performed on 1 5% agar slides according to *Grabar* (6) Double immunodiffusion for 72 h at 4°C was performed with Ouchterlony s technique in 1 5% agar (6 19) Crossed immunoelectrophoresis was performed according to *Axelsen et al* (12)

SDS polyacrylamide Gel Electrophoresis

Protein Concentration

The concentration of protein was calculated using extinction coefficients at 280 nm

$$E^{1\%}_{1\,cm}$$

of 13 5 and 15 0 for the intact antibody and the Fab fragments respectively (9)

RESULTS

Purification of Renin Specific Fab Fragments

The purification of renin specific Fab fragments were carried out with special emphasis to avoid Fc fragments and undigested antibodies

The IgG fraction containing specific antibodies was isolated from sera of the immunized rabbits on a DEAE-column (8) The purification step was followed by immunoelectrophoresis with polyspecific hog antiserum against rabbit serum proteins The immunoelectrophoresis showed only a single precipitation line corresponding to the mobility of IgG The gelfiltration of a papain digest of IgG on Sephadex G 100 is shown in Fig 1 Two peaks appeared a small one corresponding to the position of undigested antibodies (mol wt 150 000) (8) The main peak contained Fab and Fc fragments (mol wt

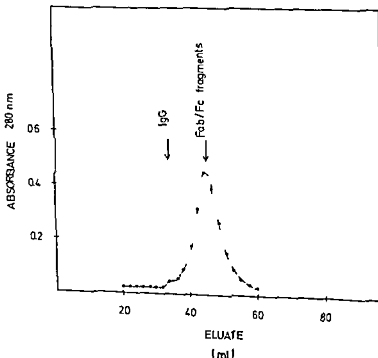


Fig 1 Gelfiltration of a papain digest of IgG on Sephadex G 100

0 5 ml of Freunds complete adjuvant The injections were repeated each month High titre antibodies were obtained after 8 weeks from 3 out of 4 rabbits A quantity of 25 ml blood could be drawn from the ear vein every 3 weeks The plasma (EDTA) was stored at − 20°C

Purification of Rabbit IgG on DEAE Cellulose

DEAE cellulose DE 52 (Sigma) preswollen was equilibrated in 10 mM phosphate buffer pH 8 0 Plasma (10 ml) from the immunized rabbits was dialyzed 18 h against the starting buffer and applied to a 1 5 × 20 cm column of DEAE cellulose and eluted with the starting buffer

The first eluted peak of 60 ml containing rabbit IgG including submaxillary renin specific antibodies (8) was pooled and ultrafiltrated (Millipore) to an appropriate volume 2–3 ml

Papain Digestion of Rabbit IgG

The ultrafiltrated gammaglobulin fraction was exposed to digestion with papain according to the method of *Nisonoff* (16) in a reaction mixture containing 2 mM EDTA and 10 mM cysteine in 0 1 M phosphate buffer pH 7 4 Papain (Mercuripapain Sigma) containing 25 mg/ml was added dropwise while shaking the reaction mixture The amount of papain added was based on that of globulin the ratio used was 1/100 (w/w)

After 3 h of digestion at 37°C the mixture was placed in an ice water bath and iodoacetic acid freshly dissolved in 0 1 M Na_2HPO_4 was added to a final concentration of 15 mM After 2 h at 0°C the solution was dialyzed overnight against 20 mM phosphate buffer pH 7 2 at 4°C

In order to separate cleaved IgG from uncleaved the mixture was subjected to gel filtration on Sephadex G 100 (0 5 × 100 cm) equilibrated with 20 mM phosphate buffer pH 7 2 and standardized with porcine IgG (Nat Bioch Corp Cleveland) The absorbance of each fraction was determined at 280 nm The first peak containing IgG eluted from this column was discarded and to the second peak containing Fab and Fc fragments was added 0 5% human albumin (Danish Serum Institute)

Purification by Affinity Chromatography of Mouse Renin Specific Fab Fragments

Two grams of activated CH Sepharose 4B ... on and washed on a sintered glass ... the final step of purification obtained according to *Malling & Poulsen* (11) were used as a ligand The pooled fractions containing 1 38 mg pure submaxillary renin were adjusted to 10 ml with coupling buffer which was 0 1 M $NaHCO_3$ pH 8 0 containing 0 5 M NaCl and mixed with the moist resin The mixture was placed in a stoppered vessel and rotated end-over-end for 3 h at 4°C The resin was then washed with the coupling buffer and allowed to react with 0 1 M Tris buffer pH 8 0 for 2 h at 4°C The resin was washed with three cycles of 0 1 M acetate buffer pH 4 0 containing 1 M NaCl alternating with 0 1 M Tris buffer pH 8 0 containing 1 M NaCl Finally the resin was washed with 20 mM phosphate buffer pH 7 2 containing 0 5% human albumin and 0 03% azide A column of submaxillary renin CH Sepharose 4B (0 5 × 3 0 cm) was packed and equilibrated with 20 mM phosphate buffer pH 7 2 containing 0 5% human albumin and 0 03% azide In order to prepare a control column two mg of purified human albumin (Behringwerke A G) was used as a ligand The procedure was exactly the same as that for coupling of submaxillary renin A column of the same size and equilibrated with the same buffer was used

The Fab and Fc containing peak from the Sephadex G 100 column was applied to the affinity column After application the column was washed extensively with 20 mM phosphate buffer pH 7 2 followed by the same buffer containing 1 0 M NaCl The column was turned upside down so that the elution of Fab fragments from the affinity column was performed in the opposite direction to the sample application with 20 mM citrate buffer pH 3 0 The elution profile was followed by absorbance at 280 nm and the inhibitory capacity of Fab fragments

Determination of the Inhibitory Capacity of Fab Fragments

In order to measure the Fab fragments inhibition of renin s enzymatic activity the radioimmunoassay measuring renin by antibody trapping of the angiotensin I formed was used (21) At 4°C 10µl of pure submaxillary renin containing 125–1000 pg was mixed with 10 µl of an appropirate dilution of Fab fragments in 0 1 M phosphate buffer pH 7 2 or as a control 10 µl 0 1 M phosphate buffer pH 7 2 and stored for 24 h at 4°C in order to approach equilibrium (11) At the end of the incubation rat renin substrate and angiotensin I antibodies (21) were added and the mixture was incubated at 37°C for ½ 1 h depending on the renin concentration To this mixture was at 4°C added 1000 µl labelled angiotensin I containing about 4000 cpm The mixture was incubated for 18 h at 4°C The separation of free and bound angiotensin I was carried out by simultaniously addition of charcoal to the samples followed by immediately centrifugation at 3000 × g for 20 min at 4°C Finally the supernatant was decanted and counted (21) The data for the samples containing both renin and Fab fragments were compared with similary incubated samples without Fab fragments

One antirenin unit (AU) 50% inhibition of 1 0 × 10⁻³ GU renin (The concentration of mouse renin was established by comparison with the hog renin standard (65/119) from the Institute for Medical Research Holly Hill London)

The Direct Radioimmunoassay Procedure for Renin

The direct radioimmunoassay for renin (11) was used in order to determine the association constants for interaction of renin with rabbit renin specific antibodies and Fab fragments At 4°C 25 µl standard containing 20–10 000 pg submaxillary renin was mixed with 10 µl

olumn with purified human albumin attached to H Sepharose 4B showed that continuously appli cation of 0 148 mg/ml Fab and Fc fragments was not retained but eluted just after void volume indicating no sign of non-specific absorption to the spacer of the affinity column

Characteri ation of Isolated Fab Fragments

The isolated Fab preparation was examined by SDS polyacrylamide electrophoresis immunoelec trophoresis and immunodiffusion

SDS polyacrylamide gel electrophoresis (7 5%) of the isolated Fab fragments containing 20 µg respectively revealed only a single band in the expected zone (N = 2) No band in the gammaglobu lin zone was detectable (Fig 3) Crossed immunoe lectrophoresis of 2 5 µg or 5 µg purified Fab fragments was performed in 1 5% agarose The Fab preparation gave only rise to a single precipita tion line in a gel containing 7 µl/cm² polyspecific hog antiserum against rabbit serum proteins (N = 3)

In order to directly detect impurities of Fc fragments in the isolated Fab preparation immuno-diffusion was performed against horse anti rabbit Fc fragments

Five µg of the isolated Fab preparation and 5 µg of a mixture of Fab and Fc fragments from the Sephadex G 100 column (Fig 1) were applied to the agarose gel plate No detectable precipitation was seen corresponding to the purified Fab preparation while the Fab and Fc preparation showed a distinct precipitation line (N = 2)

Association Kinetics

Association kinetics of renin specific Fab frag ments and antibodies were determined by the direct radioimmunoassay for renin (6) The procedure was carried out at 4°C The result of determination of average association constants for interaction of renin with rabbit renin specific antibodies and Fab fragments are shown in Fig 4 The Scatcard plot shows a K_a value for the undigested antibodies of $1\ 0 \times 10^{11}$ l/mol and for the Fab fragment a K_a value of $0\ 7 \times 10^{11}$ l/mol In order to compare the affinity of Fab fragments for the antigenic determi nants of renin and for the enzymatic inhibition the Fab fragments inhibitory capacity on renin s enzy matic activity (see methods) was used to calculate the affinity The calculated association constant for the enzymatic inhibition of the Fab fragments was $0\ 5 \times 10^{11}$ l/mol

The affinity of the purified Fab fragment for higher molecular weight forms of renin was determined by immunoreactivity and by enzymatic inhibition The purified Fab fragment and the uncleaved IgG antirenin reacted with high molecu lar weight forms exactly in the same way and the results were identical to that of *Malling & Poulsen* (12)

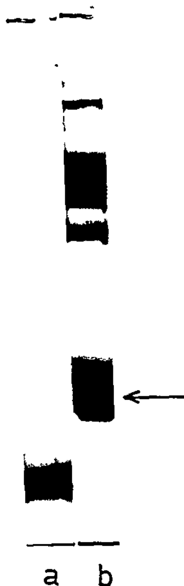


Fig 3 SDS-polyacrylamide gel electrophoresis (7 5%) showing a single band of the purified Fab fragments a Purified Fab fragments 20 µg protein was applied to the gel (2 mercaptoethanol was omitted) b Impure preparation of human albumin (arrow) 40 µg protein was applied to the gel (incl 2 mercaptoethanol)

50 000) (8) In Fig 2A it is demonstrated that continuously application of 0 148 mg/ml Fab and Fc fragments are retained on the affinity column with submaxillary renin attached The limit of the affinity columns capacity is reached at 100 ml eluate corresponding to a capacity of 9 86 mg Fab fragments Arrow B in Fig 2A indicates the ending of application of Fab and Fc fragments followed by elution with 20 mM phosphate buffer pH 7 2 In order to eliminate nonspecific absorption the column was washed extensively with 20 mM phosphate buffer pH 7 2 containing 1 0 NaCl indicated as arrow C in Fig 2A Desorption from the column of the applied renin specific Fab fragments was carried out by adjustment to pH 3 0 and was performed in the opposite direction to sample application The desorption buffer was 20 mM citrate buffer pH 3 0 indicated as arrow D in Fig 2A The desorption eluate was followed by absorbance at 280 nm and when it approxim... zero the column was washed with 20 mM phosphate buffer pH 7 2 (arrow E Fig 2A) Attempts to obtain higher recovery by usi... chaotropic ions (16) were made by exposing th... affinity column to 2 0 M KJ at neutral pH (arrow F Fig 2A) The column was immediately washed with 20 mM phosphate buffer pH 7 2 The recovery after acid elution was approximately 85–90% and after reuse there was no detectable decrease in affinity and the recovery was still 80–85% (N 4) The increase in recovery by using chaotrop... ions was raised by only 2–3% (N = 2) Stepwise elution with 4 6 and 8 M urea showed only further increas... in recovery of 2% (Fig 2A) In Fig 2B a control

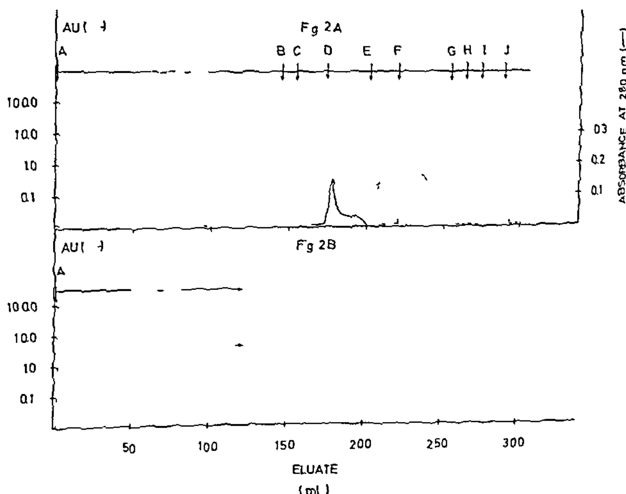


Fig 2 column follow... 7 2 + 0 5% albumin B Elution with 20 mM phosphatebuffer pH 7 2 C The same buffer with addition of 1 0 NaCl D Elution with 20 mM citrate buffer pH 3 0 E The same elution buffer as B F Elution with 2 0 M KJ in mM Trisbuffer pH 7 2 G H I and J Elution with 4 6 and 8 M urea followed by 20 mM phosphate buffer pH 7 2

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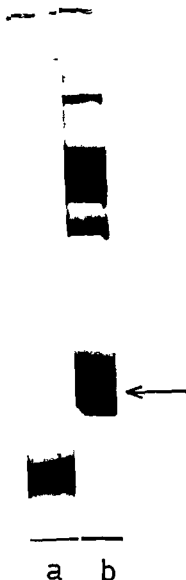


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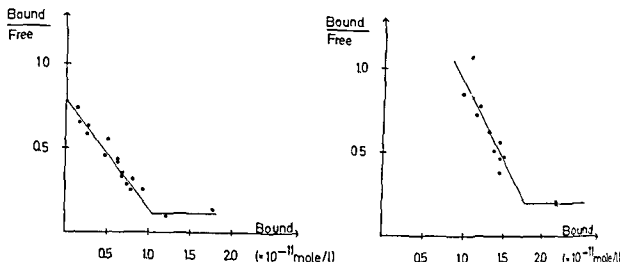


Fig 4A Scatcard plot of the purified Fab fragments reaction with renin B Scatcard plot of the antibody preparation from which the Fab fragments was purified The K_a value is calculated from the slope of the curves

DISCUSSION

Purification of renin specific Fab fragment was performed in order to obtain a specific inhibitor of renin avoiding aggregation or precipitation of the antigen antibody complex in vivo and avoiding binding of complement of the complex with secundary release of histamine *Poulsen* (20) showed that antisera against hog renin bound complement when reacted with renin The binding site of complement is localized to the first domain of the Fc fragment of the antibody (3) The complement binding releases histamine an could therefore cause an anafylactoid reaction which would decrease the bloodpressure if the antigen antibody reaction took place in vivo The decrease in bloodpressure elicited by the histamine release would be misinterpreted as being inhibition of renin s enzymatic activity by antibodies (5) The antisera raised against pure renin (11) showed complete inhibition of the enzymatic activity of renin in vitro A difference in the inhibitory capacity of the Fab fragments compared to the divalent antibodies was not detectable supporting the conclusion that aggregation does not play a major part in the inhibition of enzymes (13)

The association constant for the divalent antibody determined by the direct radioimmunoassay for renin was $1\ 0 \times 10^{11}$ l/mol and comparable to that by *Malling et al* (11) who found a K_a value of $2\ 0 \times 10^{11}$ l/mol The association constant for the reaction between renin and the divalent antibody compared to renin and the purified Fab fragments (from the same antibody preparation) showed no difference The results are in agreement with those found by *Nisonoff et al* (17) studying the binding of hapten to purified rabbit anti p azobenzoate IgG and its Fab fragment The association constant for the antigenic determinants and the enzymatic inhibition was practically the same $0\ 7 \times 10^{11}$ l/mol and $0\ 5 \times 10^{11}$ l/mol indicating that the antigenic determinants at the surface of the renin molecule must be in close contact with the enzymatic site or localized in a position with possibility of steric hindrance of the renin substrate for the enzymatic site

So far the most potent inhibitor of renin was pepstatin an aspartylproteases inhibitor with an inhibitor constant about 10^{-6} M for renin (18) The calculated inhibitor constant the inverse of the association constant of the Fab fragments determined by measuring the enzymatic inhibition of renin was $1\ 5 \times 10^{-11}$ M This implies that the Fab fragment has a K_i value which is approximately five order of magnitude higher than the K_i value of pepstatin indicating that the pure Fab fragment is the most potent inhibitor of mouse renin available The affinity of the purified Fab fragment and the uncleaved IgG antirenin for higher molecular weight forms of renin were identical The pure renin specific Fab fragment will be used to determine the possible role of renin in renal hypertension

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UPTAKE OF NON-OPSONIZED *E COLI* BY UNSTIMULATED MOUSE PERITONEAL MACROPHAGES

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Rollag H Uptake of non-opsonized *E coli* by unstimulated mouse peritoneal macrophages Acta path microbiol scand Sect C 87 99–105 1979

A method is described for the study of phagocytosis of ^{32}P labelled non-opsonized viable *E coli* by mouse peritoneal macrophages Factors influencing the uptake such as medium number of bacteria and time of phagocytosis were standardized The kinetics of the uptake were studied by visual counting of bacteria and by measuring the distribution of radioactive labelling The uptake of ^{32}P by the macrophages is well correlated to the number of bacteria phagocytized The amount of phagocytosis depends on the bacterial density of the medium and the time of phagocytosis When the medium contains more than 10^9 bacteria per ml the maximum phagocytic capacity is reached after 90 minutes

Key words Mouse peritoneal macrophages *E coli* phagocytosis

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The phagocytic activity of macrophages is an important part of the host defense The mechanisms of phagocytosis by these cells are only partly known There are variations between different types of macrophages and even within the same type there are different pathways (3 10) Macrophages have at least three functional receptors for attachment and subsequent ingestion of particles viz the Fc receptor the complement receptor and the non specific receptor (11) The selection of receptor is a function of the particle initiating the phagocytic response [illegible]
ment fact[illegible]

Phagoc[illegible] been studied using mostly inert particles and the knowledge regarding living bacteria is limited

This paper describes the phagocytosis of ^{32}P labelled v able non ops[illegible] phagocytic system for the study of some kinetic aspects of the phagocytosis of living bacteria via the non-specific receptor

MATERIALS AND METHODS

Animals

Male Ham/ICR/CSE/Bom albino mice were used in all experiments They were 1–3 months old and weighed 20–25 g

Bacter a

[illegible] the cultivation and labelling of the bacteria and the *preparation* of the bacterial suspension were performed as by *M dived & Melby* (6)

The dens ty of the bacterial suspension was $10^9 \pm 0.5$ bacteria per ml where not otherwise stated

The bacteria were labelled with ^{32}P orthophosphate

(code P 3B, Institutt for Atomenergi Kjeller, Oslo, Norway) Bacterial suspensions were made in Krebs Ringer phosphate buffer with 10mM glucose (KRG), pH 7 4, prepared according to *Roberts & Quastel* (9)

Harvesting and Processing of Mouse Mononuclear Cells

Mice were killed by cervical fracture Mouse peritoneal macrophages (MPM) were obtained by a method described by *Degre* (3) Three ml ice-cold Eagles minimal essential medium with Hanks' salts (EH Grand Island Biological Company, New York) supplemented by heparin 5 I U per ml (Nyco, Norway) and 200 µg per ml oxytetracycline (Dumex, Denmark) were injected into the peritoneal cavity of mice without previous stimulation After light massage the fluid was withdrawn Cells from 10–20 mice were pooled centrifuged at 120 × g for 5 min and resuspended in EH medium containing 20 per cent inactivated (56°C for 30 min) foetal bovine calf serum (FBS Flow Laboratories) 0 132 per cent $NaHCO_3$ and antibiotics as above

The cell suspension was adjusted to $1-2 \times 10^6$ cells per ml and the pH of the suspension was 7 4

Aliquots of 1 0 ml of this suspension were distributed into Leighton tubes containing a 11 × 35 mm »flying coverslip« and incubated for 2 h in a humid atmosphere with 5 per cent CO_2 The tubes were then agitated vigorously The supernatant fluid was discarded and the glass adherent cells were washed once with EH medium The above mentioned culture medium but without antibiotics was then added and the cells were incubated for 24 hours

The cells were identified as macrophages firstly by light microscopy and secondly by their ability to phagocytize carbon particles Four hundred cells in three different preparations were counted

Performance of the Phagocytosis Experiments

The cells on coverslips were incubated in EH medium with 20 per cent FBS for 24 h The coverslips were removed aseptically washed twice in physiological saline solution at 37°C to remove serum The coverslips were then put into new Leighton tubes containing the prewarmed (37°C) bacterial suspension The tubes were incubated in humid atmosphere with 5 per cent CO_2

After phagocytosis periods of 30 60 90 or 120 min the coverslips were removed washed three times in physiological saline solution at 37°C to remove glass adherent bacteria and non macrophage bound radioactivity After drying the coverslips were put into tubes with 1 2 ml 0 1 N NaOH where the macrophages were detached and dissolved

Aliquots of 0 2 ml were removed for ^{32}P determination and the rest (1 0 ml) were used for cell protein determinations

The samples for ^{32}P determination were transferred to a scintillation vial containing 10 ml Aerosol (Koch Light Laboratories) The samples were counted in a »Packard« liquid scintillation counter for 10 min

The protein determinations were performed according to the *Oyama & Eagle*'s modification of the method of *Lowry et al* (8)

The rate of phagocytosis was expressed as counts of ^{32}P radioactivity per mg cell protein and per minute (CPMM) The experiments were carried out in at least three parallel runs

In most experiments, duplicate coverslips were fixed in 96 per cent ethanol and stained with Giemsa stain The number of bacteria was counted in a light microscope

Release of Label from Bacteria to Medium

Leighton tubes with 10^6 bacteria per ml of ^{32}P labelled *E coli* suspended in KRG medium were incubated in a humid atmosphere with 5 per cent CO_2 without the presence of macrophages 0 1 ml of the suspension was removed after various times for determination of the total ^{32}P activity in the tubes The rest was centrifuged at 15 000 × g and 2°C for 10 min After centrifugation 0 1 ml of the supernatant fluid was removed for determination of ^{32}P not bound to the bacteria

The release of ^{32}P to the medium was expressed as the ratio of ^{32}P in the supernatant to the total ^{32}P activity of the *E coli* suspension

Uptake in the Macrophage of ^{32}P not Associated with the Bacteria

The phagocytosis experiments were performed as described above The unlabelled bacteria were suspended in KRG medium containing ^{32}P activities of 0 10^2 10^3 10^4 10^5 and 10^6 counts per ml and per minute The uptake of ^{32}P from the medium during the phagocytosis of the unlabelled bacteria was determined after phagocytosis periods of 30 60 and 90 minutes

Release of ^{32}P to the Medium from Macrophage associated Bacteria

The phagocytosis experiments were performed as described above After 60 minutes of phagocytosis the coverslips were removed aseptically and washed three times with physiological saline They were then put into new Leighton tubes with 1 0 ml of KRG medium and incubated again at 37°C At various times the coverslips were removed and the ^{32}P activity determined The medium from the tubes was immediately cooled to 0°C and the ^{32}P activity before and after centrifugation at 15 000 × g and 2°C for 10 minutes was determined

The release of ^{32}P to the medium was calculated from the quotient

$$\frac{^{32}P \text{ in the medium after centrifugation} \times 100\,\%}{^{32}P \text{ on the coverslip} + {}^{32}P \text{ in the medium before centrifugation}}$$

RESULTS

The tables and the illustrations are based on the results of selected typical experiments Experiments were carried out three to four times and each with at least three parelle| runs

Fate of E coli *in the KRG Medium*

The growth of *E coli* and its ability to retain the ^{32}P marker under the experimental conditions were tested Growth of *E coli* (10^9 CFU/ml) was measured as variations in CFU and O D $_{660}$.

During the 120 min the CFU increased from 2 8 to 3 2 × 10^9 CFU/ml whereas the O D $_{660}$ increased from 0 41 to 0 42 The growth curves are shown in Fig 1

At the start of the phagocytosis experiments the total ^{32}P activity was 10^6 counts per minute and per ml (± 25 per cent) More than 97 per cent of this activity was associated with the bacteria Fig 2 shows the loss of ^{32}P from *E coli* to the medium when the bacteria were grown in KRG medium in the absence of macrophages

After incubation for 120 minutes the number of bacteria had increased about 15 per cent but still 97 per cent of the ^{32}P activity of the suspension was bound to bacteria

Macrophages

On the basis of the criteria given more than 98 per cent of the glass adherent cells could be classified as macrophages By counting 25 random

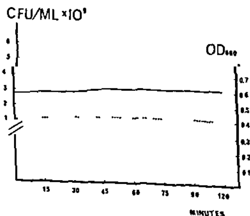


Fig 1 Growth of *E col* in KRG
Growth
(——) a
(OD$_{660}$)(

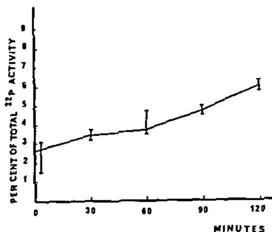


Fig 2 Release of ^{32}P from radiolabelled *E coli E coli* (10^9 bact /ml) labelled with ^{32}P was grown in KRG medium at 37° C After various times suspensions were centrifuged at 15000 × *g* and 2° C for 10 minutes The release of ^{32}P from bacteria to the medium is the percentage of radioactivity in the supernatant fluid after centrifugation to the total radioactivity of the bacterial suspension before centrifugation The figure presents the mean and range of three parallel runs

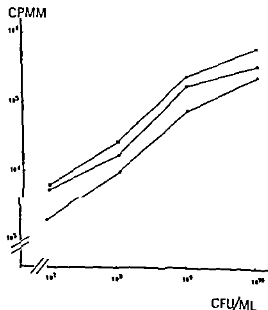


Fig 3 Effect of bacteria macrophage ratio on phagocytosis Mouse peritoneal macrophages on coverslips in Leighton tubes were exposed to ten fold dilutions of 0 8 × 10^{10}
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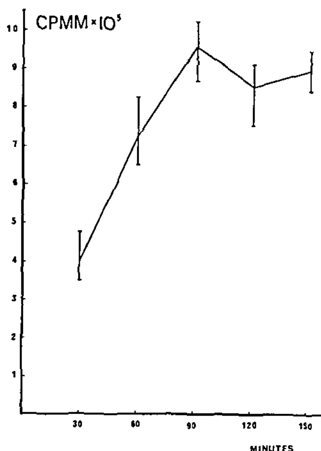


Fig 4 Phagocytosis of *E coli* by MPM after various periods of incubation Mouse peritoneal macrophages (MPM) were cultivated on glass coverslips in Leighton tubes for 24 h They were then exposed to 32P labelled *E coli* (1 4 × 10^9 bact /ml) for 30 60 90 and 120 minutes The rate of phagocytosis is expressed as counts per mg cell protein and per minute (CPMM) Each point on the curve is the mean of five parallel runs the vertical bars represent the range

sight fields or by measuring the protein content of glass adherent cells the number of macrophages per coverslip varied between 2 to 5 × 10^5

Influence of various Bacteria Macrophage Ratios on the Phagocytosis

Macrophages were incubated with 32P labelled *E coli* suspensions with densities ranging from 0 8 × 10^7 to 0 8 × 10^{10} bacteria per ml Phagocytosis was determined after 30 60 and 90 minutes The steepest increase in the rate of phagocytosis was 0 8 × 10^8 and 0 8 × 10^9 bacteria per ml By increasing the number of bacteria from 0 8 × 10^9 to 0 8 × 10^{10} per ml there was only a two to three fold increase in the phagocytosis (Fig 3)

On the basis of these findings dilutions containing 10^9 *E coli* per ml were used in the subsequent experiments This gave a bacterium macrophage ratio of 10^3 1 or more

Uptake of 32P labelled E coli *by MPM after various Times of Phagocytosis*

The kinetics of Phagocytosis of 32P labelled *E coli* are presented in Fig 4 During the first 90 min there was a linear increase in the uptake of 32P The maximum phagocytic capacity was reached after 90 min and there was no further increase during prolonged incubation time

Reproducibility of the Phagocytosis System

Repeated experiments indicated that the uptake of 32P labelled *E coli* by MPM was highly reproducible provided that the number of bacteria and cells the medium and the experimental conditions were kept constant

The reproducibility was similar after the different periods of phagocytosis (Table 1)

Correlation between 32P activity and Number of Bacteria per Macrophage after various Times of Phagocytosis

The mean number of *E coli* per macrophage and the 32P activity per mg cell protein was determined on parallel coverslips after 30 60 and 90 minutes of phagocytosis The increase in the number of

TABLE 1 *Reproducibility of the Phagocytosis System*

Phagocytosis time	No of parallels	Mean 32P activity (CPMM)	Range (CPMM)	S D (CPMM)
30 min	13	50 5 × 10^3	44– 60 × 10^3	6 5 × 10^3
60 min	16	131 × 10^3	100–167 × 10^3	21 × 10^3
90 min	19	242 × 10^3	188–349 × 10^3	38 × 10^3

Mouse peritoneal macrophages on glass coverslips in Leighton tubes were exposed to 32P labelled *E coli* (10^9 bact / ml) The rate of phagocytosis after various exposure times is expressed as counts per mg cell protein and per minute (CPMM)
Variations in the uptake are given as range and one standard deviation (S D)

TABLE 2 *Phagocytosis of ^{32}P labelled* E. coli *by MPM. Relationship between Uptake of ^{32}P and the Number of Bacteria per Macrophage*

Phagocytosis time	30 min	60 min	90 min
^{32}P activity (CPMM)	74×10^3 ($61–86 \times 10^3$)	232×10^3 ($210–250 \times 10^3$)	358×10^3 ($320–390 \times 10^3$)
No of *E. coli* per macrophage	4.2 (3.6–4.8)	12.6 (10.3–15.0)	23.0 (20.3–26.2)

Mouse peritoneal macrophages were exposed to ^{32}P labelled *E. coli* (10^9/bacteria/ml) after incubation for 24 hours.

The rate of phagocytosis after exposure times of 30, 60 and 90 min was measured as the uptake of ^{32}P (CPMM) and as the mean number of visible bacteria per macrophage. The experiment was performed in six parallel runs, three for estimation of the ^{32}P activity and three for visual counting of the number of bacteria per macrophage. The range of the findings on duplicate coverslips is given in brackets.

bacteria phagocytized correlated well with the increased uptake of ^{32}P (Tabel 2).

Uptake of ^{32}P from the Medium during Phagocytosis of Unlabelled E. coli

The uptake of ^{32}P in solution measured at various times and with various concentrations of ^{32}P is negligible, as shown in Table 3.

Release of ^{32}P from Macrophage associated Bacteria to the Medium

Release could be expressed in two ways, firstly as a decrease in the macrophage associated ^{32}P activity and secondly as the percentage of ^{32}P released from the macrophages to the medium. The degradation starts immediately after uptake in the macrophage (Table 4).

DISCUSSION

The method in the present study was established in order to examine the phagocytosis of viable bacteria by the non specific receptor into mononuclear phagocytes. A further aim was to use the procedure

TABLE 3 *Uptake of ^{32}P from the Medium by MPM during Phagocytosis of unlabelled Bacteria*

Time of phagocytosis	^{32}P activity in the medium[a]	Uptake of ^{32}P from the medium into the MPM (CPMM)
30, 60, 90 min	10^4	Not detectable
30, 60, 90 min	10^5	Not detectable
90 min	10^6	8.6×10^3

[a] Counts per minute and per ml

Mouse peritoneal macrophages (MPM) were exposed to suspensions of unlabelled *E. coli* (10^9 bact./ml). The suspension medium was supplemented by various concentrations of ^{32}P orthophosphate. The amount of ^{32}P taken up from the medium by the macrophages during the phagocytosis of unlabelled *E. coli* was measured and expressed as counts per mg cell protein and per minute (CPMM).

TABLE 4 *Release of ^{32}P from the Macrophage associated Bacteria to the Medium*

Time	^{32}P activity per coverslip (CPMM)	Percentage of total ^{32}P activity released to the medium
0 min	165×10^3 100%	0%
30 min	148×10^3 90%	13%
60 min	127×10^3 77%	28%
90 min	116×10^3 69%	32%
120 min	79×10^3 48%	42%

Mouse peritoneal macrophages (MPM) were exposed to ^{32}P labelled *E. coli*. Following uptake periods of 60 min

to investigate the influence of external factors on the uptake of bacteria. The use of viable bacteria will make it possible to develop the method for further studies on the subsequent steps in the intracellular fate of phagocytized bacteria.

Interaction between two living organisms such as mouse peritoneal macrophages and *E. coli* should be studied under culture conditions optimal for both. The KRG medium described by *Roberts & Quastel* seemed to be favourable for both the macrophages and the bacteria used in this study (9). Using this method and a humid atmosphere with 5 per cent CO_2, the macrophages retained their phagocytic capacity for at least 2 h. The protein content of macrophages on coverslips did not show any significant variations during the periods of phagocytosis (results not shown). Giemsa-stained macrophages in coverslips did not show any morphological changes, even after cultivation for 4 h in KRG medium.

The long periods of phagocytosis used in our experiments require suspension medium that restricts growth of the microorganism. It is of importance that the radioactive marker is not released from the bacteria to the medium. When ^{32}P-labelled *E. coli* (10^9 bacteria per ml) is cultivated in KRG medium for 150 min, the number of bacteria increases only about 15 per cent, and during the same period only 6 per cent of the bacteria-bound ^{32}P is released to the suspension medium. Therefore, both the number of viable *E. coli* and the ^{32}P label are sufficiently stable during the experimental periods when suspended in KRG medium. The same medium seems to be sufficient to maintain the functional activities of macrophages.

Our intention was to study the uptake of bacteria with a defined and constant bacteria-macrophage ratio. To avoid significant elimination of bacteria from the medium during the experimental period due to uptake into the macrophages, we employed a high number of bacteria giving a ratio of 1000:1. This ratio was kept fairly constant during the observation period.

Release of radioactivity from the bacteria and from the phagocytic cell after uptake of bacteria may be an important source of error in an assay system. When ^{32}P-labelled *E. coli* is cultivated in KRG medium, more than 94 per cent of total ^{32}P is bound to bacteria (Fig. 2). The uptake of this soluble ^{32}P during the phagocytosis is negligible (Table 3). *Cohn* found that the degradation and release of [illegible] starts immediately after [illegible] macrophages [illegible] finding. This may lead to underestimation of the amount of phagocytosis, although in the present system this factor seems to be of minor importance. A parallel increase in the uptake of ^{32}P and the number of bacteria per macrophage during the period of phagocytosis, as shown in Table 2, indicates that the cell bound ^{32}P is a dependable correlate for the phagocytic activity.

Due to qualitative differences as well as variations in the number and degree of labelling of the bacteria from one experiment to another, direct comparison of data between experiments is difficult. Each experiment must include its own controls.

The rate of phagocytosis and the maximum phagocytic capacity are determined to a great extent by the adhesiveness between the particle and the macrophage, and how rapidly the particle becomes internalized (2, 4, 5). Particles with a high hydrophobicity attach easier (2). Data on the kinetics of phagocytosis are therefore valuable only for the phagocytic cell and the microorganism used in that particular study. The results presented in this study concern the kinetics of *E. coli* phagocytized by MPM, and are not necessarily applicable to other systems. Electron microscopy showed that attachment of bacteria to the macrophage was followed by engulfment of the bacteria (to be published later).

The density of the bacterial suspension was crucial for the number of bacteria taken up during a period of phagocytosis (Fig. 3). When using 1.4 × 10^9 bacteria per ml, the total phagocytic capacity was reached after 90 minutes of phagocytosis (Fig. 4). If bacterial suspensions with less than 10^9 bacteria per ml were used, the total phagocytic capacity was not reached after 90 minutes of phagocytosis. The rate of phagocytosis, i.e. the speed with which the particles are taken up by the macrophages, was less influenced by the density of the bacterial suspension. Using 0.8 × 10^8, 0.8 × 10^9 and 0.8 × 10^{10} bacteria per ml, there was an almost parallel increase in the uptake during the first 90 minutes of phagocytosis (Fig. 3). This corresponds well with the data obtained by *Rowley* in his study on phagocytosis of non opsonized *E. coli* (10) and by *Jones* on non-opsonized mycoplasmas by MPM (4).

This system for phagocytosis of non opsonized *E. coli* by MPM does not permit discrimination between the attachment and the engulfment phases of phagocytosis.

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EFFECTS OF ACETYLSALICYLIC ACID ON LYMPHOCYTE SUBPOPULATIONS IN PERIPHERAL BLOOD

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Sørensen S F Dirksen A Jensenius J Jønsson V Platz P & Hansen G S Effects of acetylsalicylic acid on lymphocyte subpopulations in peripheral blood Acta path microbiol scand Sect C 87 107–112 1979

The *in vitro* and *in vivo* effects of therapeutical doses of acetylsalicylic acid on lymphocyte subpopulations in peripheral blood were investigated with the following results Acetylsalicylic acid caused both *in vitro* and *in vivo* a reduction of complement receptor bearing lymphocytes and of lymphocytes identified with fluorescent rabbit antibody to human Ig (polyvalent) and to human IgG Sheep red blood cell receptor bearing lymphocytes and lymphocytes identified with antibody to human IgM and IgD were unaffected by acetylsalicylic acid

Key words Lymphocyte subpopulations acetylsalicylic acid peripheral blood

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Accepted as submitted 16 x 78

Several recent studies have been concerned with the enumeration of lymphocyte subpopulations in the peripheral blood of patients with vario is

ic acid is so widely used in the treatment of connect

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MATERIAL AND METHODS

Examination of Lymphocytes

Blood from normal healthy donors were used throughout The total number of lymphocytes was calculated from a leucocyte count and a differential count using routine techniques Lymphocytes and monocytes were isolated from peripheral blood by flotation on Lymphoprep® as previously described (18) After three washes in Hanks solution the cells were incubated in Hanks sol tion w th

(11 12)

longer than one week and washed three times in Hanks solution before use The mononuclear cells were resuspended in foetal calf serum (FCS) heated to 56° C ½ h before use and the cell concen

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cells we e resuspended by carefully rotating the tube between two hands and the fraction of rosette forming lymphocytes was determined in a conventional hemocytometer

Immunofluorescence test

The following fluorescein (FITC) conjugated IgG fractions of rabbit anti human immunoglobulin antisera were used

a) a polyvalent antiserum against IgM IgG IgA kappa and lambda light chains diluted 1 6
b) a monovalent antiserum against μ chains diluted 1 4
c) a monovalent antiserum against γ chains diluted 1 4
d) a monovalent antiserum against δ chains diluted 1 4

The three first mentioned reagents at 2–5 mg IgG/ml were obtained from Dacopatts A/S Copenhagen whereas the anti δ chain reagent was obtained from Behringwerke

e) $F(ab')_2$ fragments of the anti γ chain reagent were prepared by pepsin digestion (15) 50 mg of FITC labelled IgG anti γ chain was incubated (20 h 37° C) with 1 mg of pepsin in 3 ml 0 1 M acetate pH 4 5 centrifuged 2000 *g* 20 min and fractionated on Sephadex G200 in Tris buffered saline (0 14 M NaCl 0 01 M Tris/HCl pH7 4) The $F(ab')_2$ peak was pooled concentrated by ultrafiltration to 20 mg in 2 5 ml The $F(ab')_2$ was pure as judged from analysis by SDS polyacrylamide slab gel electrophoresis The anti γ chain reagent was diluted 1 4

Contamination of the $F(ab')_2$ with IgG Fc or pFc was also analysed by radioimmunoassay as previously described (10) with minor modifications Precipitation and wash of precipitate was carried out with 15% polyethylene glycol on glass fiber filters rather than with ammonium sulphate

The assay was constructed with radioiodinated rabbit pFc horse anti rabbit IgG antiserum and rabbit IgG as inhibitor for standard curve Analyzed by this assay the $F(ab')_2$ preparation was found contaminated with Fc corresponding to about 0 5% (w/w) of IgG

The final dilution of each antiserum was selected on the basis of preliminary titration experiments as the dilution yielding the maximal number of stained cells

The cell suspensions were centrifuged and three drops of each antiserum dilution were added to the cell buttons in each tube The tubes were maintained at 0° C for 30 min After three washes in Hanks solution with 5% human albumin at 4° C the cells were resuspended in two drops of a 1 4 mixture of Hanks solution and glycerol At least 200 lymphocytes in each preparation were examined for the determination of the fraction of lymphocytes with membrane fluorescence

Quantitation of Fc Receptor Bearing Cells

To 10 ml of blood 0 58 g of carbonyl iron powder was added After slowly rotation of the test tube for 20

[illegible]

tested in 9 experiments by incubating the cell suspensions for 1 h at 37° C with latex particles in an 1 1 mixture of FCS and Hanks solution Usually no labelled cells were found but in a few experiments 0 1–0 4 per cent latex ingesting cells were found

For the rosette test the mononuclear cells were isolated and processed as described above except during the one hour incubation at 37° C latex particles were omitted

EA marker cells were prepared by incubating 4 ml one per cent (v/v) SRBC (E) with 4 ml IgG antibody reagent (A) at 37° C for 20 min IgG reagent was obtained from rabbits after injections with SRBC during a period of 42 d The serum was collected and the IgG was isolated on a DEAE Sephadex A 50 column (7) After heat inactivation at 56° C for 30 min and after treatment with 2 mercaptoethanol the IgG reagent was diluted to ¼ agglutinating unit One agglutinating unit was defined as the amount of the highest dilution of antiserum which agglutinates an equal amount of a 1 per cent suspension of E (19)

For rosetting 0 1 ml of a 1 per cent (v/v) EA marker cell suspension was mixed with 0 1 ml lymphocyte suspension (4×10^6/ml) The cells were centrifuged at 200 G for 3 min and incubated at 37° C for 20 min After resuspension of the cells on a Wortex mixer the number of rosettes per 200 lymphocytes was counted Lymphocytes with 3 or more erythrocytes were scored as rosettes

Quantitation of Complement Receptor Bearing Lymphocytes

The lymphocyte suspension was prepared as for the estimation of Fc receptor bearing cells

Antibody complement coated erythrocytes (EAC) were prepared ad follows Human A erythrocytes washed 3 times with Hanks solution and resuspended to a 2 5 per cent (v/v) solution were incubated 30 min at 37° C with an equal volume of an IgM rabbit anti A serum diluted to optimal non agglutinating concentration (1 2000) 0 1 ml mouseserum (complement source) was added to 2 ml of the erythrocyte suspension in 30 min at 37° C and finally diluted with 3 ml Hanks solution

For rosetting 0 1 ml EAC suspension was mixed with 0 1 ml lymphocyte suspension (3×10^6/ml) and centrifuged for 5 min at 72 G After 5 min the pellet was gently resuspended and the number of rosettes per 200 lymphocytes was counted Lymphocytes with 3 or more erythrocytes were scored as rosettes

Administration of Acetylsalicylic Acid

In the *in vitro* experiments the cell suspension was divided in two alliquots with approximately 10^7 cells in two ml each Acetylsalicylic acid was added in the form of lysine acetylsalicylate (Aspegic®) in a final concentration of 30 mg/100 ml during the one hour incubation at 37° C The results are expressed as percentage of each kind of lymphocyte subpopulation in the acetylsalicylic acid incubated suspension compared to the control suspension

In the *in vivo* experiments the blood donors were kept fasting for the first two hours At time 0 blood was collected and immedeately afterwards 2 g of acetylsalicylic acid was given orally (Magnyl®) Two hours later another blood sample was collected Finally blood was collected 24 hours after ingestion of acetylsalicylic acid from the fasting blood donor

Results of the *in vivo* experiments are given as the

alculated total concentrations of each kind of lymphocyte subpopulation in the blood

Statistical Test

The Wilcoxon test for pair differences has been used throughout

RESULTS

Figs 1 and 2 show the result of incubating lymphocytes with and without acetylsalicylic acid for one hour at 37° C *in vitro* before assay for rosette forming cells and immunoglobulinpositive cells It is seen that the percentage of complement receptor bearing lymphocytes and lymphocytes stained with the polyvalent anti Ig and anti IgG antibody is reduced after acetylsalicylic acid incubation ($p<0.05$ $p<0.01$ $p<0.01$ respectively) whereas the other lymphocyte subpopulations are unaffected by this treatment

It has been demonstrated that identification of surface IgG using whole rabbit anti IgG sera may identify not only cells with intrinsic surface IgG but also cells with Fc receptors (see below) Therefore experiments were performed where $F(ab')_2$ fragments of the rabbit anti IgG sera were used In Table 1 it is seen that the $F(ab')_2$ antibody indeed identify less than half the number of cells identified with the whole rabbit antibody but again a

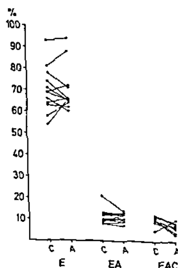


Fig 1 Effect of acetylsalicylic acid *in vitro* on SRBC R cells (E) Fc receptor positive cells (EA) and compleme-

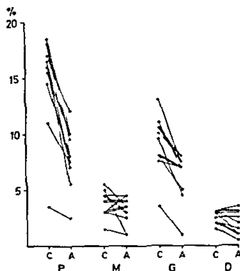


Fig 2 Effect of acetylsalicylic acid *in vitro* on cells stained with fluorescent rabbit antibody to human Ig (P) IgM (M) IgG (G) and IgD (D) See text to Fig 1

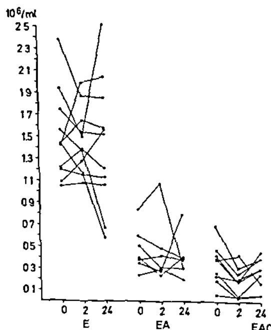


Fig 3 Effect of a...

... Two hours later another bloodsample was collected Finally blood was collected 24 hours after ingestion of acetylsalicylic acid from the fasting blood donor Values are given as absolute numbers of the various lymphocyte subpopulations

TABLE 1 *Effect of Acetylsalicylic Acid (A) in vitro on per Cent Cells Stained with Fluorescent Rabbit Antibody to IgM and IgG*

		EXP NO									
		1	2	3	4	5	6	7	8	median	P
non cleaved anti IgM	C	4 5	4 5	3 0	4 0	3 0	3 5	4 5	4 0	4 5	ns
	A	3 5	5 0	3 0	3 5	3 5	3 0	5 0	4 5	3 5	
non cleaved anti IgG	C	15 5	13 0	16 0	15 0	13 0	12 0	15 0	9 5	14 0	<0 0
	A	8 0	8 0	6 5	10 0	9 0	7 0	12 5	4 0	8 0	
F(ab)$_2$ anti IgG	C	9 0	5 0	4 0	6 0	4 0	6 0	6 5	7 0	6 0	<0 0
	A	8 0	3 5	3 0	4 5	3 5	6 0	6 0	5 5	5 0	

C controls

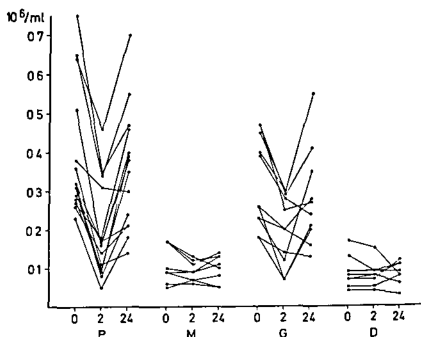


Fig 4 Effect of acetylsalicylic acid *in vivo* on cells stained with fluorescent rabbit antibody against human Ig (P) IgM (M) IgG (G) and IgD (D) See text to Fig 3

consistent fall was found after acetylsalicylic acid incubation (p<0 02)

In Figs 3 and 4 the results of *in vivo* ingestion of therapeutic doses of acetylsalicylic acid is shown Again a clearly significant fall was found in complement receptor bearing lymphocytes (p<0 05) and in lymphocytes identified with the polyvalent anti Ig and the anti IgG serum (p<0 01 in both cases) and 24 hours after acetylsalicylic acid ingestion all lymphocyte subpopulations had returned to pretreatment values Comparing the total number of lymphocytes before and 2 h after acetylsalicylic acid a mean fall of 0 25 × 10^9/l was found However this difference was not significant (p = 0 20) A similar number of experiments have been made with control individuals not ingesting acetylsalicylic acid and in no case significant deviations of lymphocyte subpopulations were found

DISCUSSION

It is well known that acetylsalicylic acid reduces *in vitro* immune responses as measured by mitogen and antigen induced lymphocyte proliferation (4 16 17) and release of leucocyte migration inhibitory

actor (3) from lymphocytes stimulated by concanavalin A To our knowledge only *Crout et al* (4) have studied the *in vivo* effects of acetylsalicylic acid on sheep red blood cell rosetting cells and cells stained with a polyvalent rabbit anti Ig serum These authors found in contrast to the present results no influence of acetylsalicylic acid on Ig positive cells This discrepancy is difficult to explain since apparently similar methods were used by these authors It is possible however that the plasma concentration of acetylsalicylic acid as employed in the present study may have been somewhat higher than in the study of *Crout et al*

The mechanisms behind the action of acetylsalicylic acid is not understood It has been reported that acetylsalicylic acid inhibits antigen antibody interactions (1) and the decreases in Ig positive and IgG positive cells observed could thus be due to cell bound acetylsalicylic acid inhibiting the interaction between human IgG and the rabbit anti IgG antibody This explanation however seems unlikely in view of the fact that IgM and IgD positive cells were unaffected by acetylsalicylic acid

incubating and washing of the cells at 37° C (as was in fact done in the present study) (11 12) and by the fact that the F(ab')₂ fragments of the rabbit anti IgG antibody identifies only very few cells in the peripheral blood (21) The mechanisms proposed to explain these findings have been that cellbound IgG is carried through the washing procedures and released when the cells are incubated with the fluorescent rabbit antibody Complexes formed between the human IgG and the rabbit antihuman IgG antibody would subsequently be bound to Fc receptor bearing cells through the Fc part of the rabbit antibody and thereby leading to a false identification of such cells as surface IgG bearing cells (11 12 21)

The apparently selective effect of acetylsalicylic acid on IgG-positive cells could thus be explained if acetylsalicylic acid acts on IgG bound to Fc receptors leading to removal of cellbound IgG The results presented in Table 1 neither exclude nor prove th[illegible]

F(ab')₂

reduce

ugh n[illegible]

whole [illegible] IgG antibody

The most straight forward explanation of the present results would be that acetylsalicylic acid acts selectively on certain lymphocyte subpopulations in the blood either by killing the cells or more likely by changing the cell-surfaces leading to blocking of complement receptors and surface IgG as identified in the techniques employed The decrease in cells identified with the polyvalent anti Ig serum could be explained by blocking of cells with surface IgG It has been shown that most cells with complement receptors in addition carry surface Ig (5 14) and in one of these studies (5) it was found that most complement receptor positive cells carry IgM and/or IgD Such results obviously depend on the methods used to identify the various lymphocyte subpopulations and it is possible that the complement receptor positive lymphocytes in the present assay include cells with intrinsic surface IgG or Fc receptors Experimental error due to contamination with monocytes were counteracted by labelling the monocytes with latex particles or removing these cells by iron ingestion before the tests However it cannot be excluded that immature monocytes escape detection due to lack of phagocytic ability Such cells carrying complement receptors and extrinsic surface IgG may thus be the target for the action of acetylsalicylic acid

The present results leave many questions open of potential interest for the anti inflammatory action of acetylsalicylic acid and further studies are in progress to explore the hypotheses mentioned above However it might be concluded that determination of lymphocyte subpopulations in various diseases with the current techniques require consideration to acetylsalicylic acid and perhaps also to other medication given to the patients at the time of the tests

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ᵀ ˙ ACROPHAGE DIFFERENTIATION
IVO AND *IN VITRO*

;es and Monocytes

Haematology and Immunology

in vitro A comparison of human
Sect C *87* 113–120 1979

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N 7000 Trondheim Norway

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HUMAN MACROPHAGE DIFFERENTIATION *IN VIVO* AND *IN VITRO*

A Comparison of Human Peritoneal Macrophages and Monocytes

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Hammerstrøm Jens Human macrophage differentiation *in vivo* and *in vitro* A comparison of human peritoneal macrophages and monocytes Acta path microbiol scand Sect C 87 113–120 1979

Human mononuclear phagocytes isolated from venous blood or sterile peritoneal exudate were cultured in an in vitro system known to [illegible]

[illegible] presumed to represent monocytes which have differentiated *in vivo* in the peritoneal exudate for 1–2 days showed several signs of increased effector cell function as compared to the relatively immature blood monocytes Cell adherence after phagocytosis ability to degrade ingested ^{125}I labelled *Candida albicans* and ability to suppress DNA synthesis in a target cell line of human origin were all found to be greater in the peritoneal cells in early culture During *in vitro* differentiation in this system both PEC and monocytes developed remarkable morphological and functional changes Cell size and granule content increased considerably Cell function measured as phagocytic digestive and cytostatic ability increased for both macrophage populations The differences between the two cell populations in early culture suggest that the functional and morphological changes induced by *in vivo* differentiation in peritoneal exudate involve changes of the same kind as those induced by *in vitro* differentiation in our system The lodging of mononuclear phagocytes in sterile peritoneal exudate does not seem to impair the capacity for further differentiation to any great extent

Key words Macrophages monocytes differentiation peritoneal exudate human phagocytosis cytostasis

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Studies on the structure and function of mononuclear phagocytes have greatly increased our knowledge of these cells over the last few years Most of these studies have been done with animal models or with *in vitro* systems using animal cells Work with human cells raises ethical and methodological difficulties that are easily overcome by the use of laboratory animals Ultimately however the mechanisms discovered in animal systems should be demonstrated in human systems in order to acquire relevance for the understanding and treatment of human disease Studies in this laboratory on human mononuclear phagocytes have demonstrated that monocytes from peripheral blood can differentiate *in vitro* into large macrophage like cells with increased lysosomal [illegible]

[illegible] differentiation is associated with the ability to suppress DNA synthesis in antigen or mitogen stimulated lymphocytes (11 12), and the ability to inhibit growth in a human cell line (13)

It thus seems that this *in vitro* culture system can provide us with human cells with macrophage characteristics for experimental study However, the relevance of functional studies on monocytes differentiated *in vitro* to human macrophage func

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$$\frac{\text{cpm supernatant}}{(\text{cpm coverslip}) + (\text{cpm supernatant}) + (\text{cpm sediment})} \times 100$$

his is referred to as digestion capacity No correction as applied for spontaneous release of isotope from the adiolabelled Candida which is about 10% for the eriod studied (16) since this will not affect the lifferences between the cell populations

Cell Adherence after Phagocytosis

Cells detaching from the coverslips during the digestion period were found in the sediment after centrifugation of the medium aspirated at harvesting The detached fraction of cells having ingested Candida was calculated as

$$\frac{\text{cpm sediment}}{(\text{cpm coverslip}) + (\text{cpm supernatant}) + (\text{cpm sediment})} \times 100$$

Macrophage mediated Cytostasis of NHIK 3025 cells

The human cell line NHIK 3025 established in 1967 from a carcinoma in situ of the cervix (7) was used as target cells The cells were maintained in RPMI 1640 with 20% human A + serum 1 glutamine and gentamycin They grow in monolayer culture in an epithelioid fashion with a mean doubling time of about 18 hours Target cells were trypsinized and 2.5×10^4 cells in 0.5 ml fresh medium were added to monocyte/PEC monolayers containing about 2.5×10^5 cells giving a target effector cell ratio of approximately 1 10 ^{3}H [illegible]

[illegible] rigorous pipetting The lysate was trapped on glass fibre filters in a Titertek multiple cell harvester (Skatron Lierbyen Norway) The filters were washed with 5% TCA left for 60 seconds and then washed for 20 seconds with water The dry filters were counted in a liquid scintillation counter (Packard Tricarb) Cytostatic index (CI) was calculated as

$$CI = 100 - \frac{(\text{cpm NHIK + effector cells}) - (\text{cpm effector cells})}{(\text{cpm NHIK})}$$

where [illegible]

[illegible] quadruplicate Variation between parallels was below 10% ^{3}H Tdr incorporation in target cells cultured without monocytes/PEC was 36 859 ± 8990 (mean ± SD of all experiments included) ^{3}H Tdr incorporation in monocytes/PEC was always below 10% of that in the target cells

Statistics

P values were obtained by Student s t test

RESULTS

Cell Separation and Culture Characteristics

In preliminary experiments to establish satisfactory separation and culture conditions for the PEC, differential counts on May-Grunwald-Giemsa stained (MGG) drop preparations of unseparated PEC showed mean values of 38% macrophages, 37% lymphocytes 24% polymorphonuclear granulocytes, and rather heavy RBC contamination By employing the method usually used for rodent PEC, with washing, plating of unseparated cells in culture medium and washing off the non adherent cells after incubation for 90 min, monolayers with 49% granulocytes, 44% macrophages and 7% lymphocytes were produced Many of the macrophages had phagocytized RBC

It has been demonstrated that endocytosis of RBC or haemoglobin interferes with macrophage mediated tumour cell killing (18) To eliminate RBC and polymorphonuclear phagocyte contamination a [illegible]

and virtually no RBC contamination 95% of the cells ingested *C albicans* thus indicating that some of the cells with lymphocyte morphology were in fact, macrophages To exclude the possibility of selection of a particular subset of macrophages at this stage drop preparations were made from different levels of tubes with Ficoll/Isopaque centrifuged ascitic fluid Virtually no cells with macrophage morphology were found outside the interface mononuclear cell band Monolayers of blood monocytes prepared as described contained 81% monocytes 10% lymphocytes and 9% granulocytes judged on the basis of MGG preparations More than 90% of the cells ingested *C albicans*

The PEC were generally larger than the blood monocytes, and many PEC contained 5–20 phase dense granules which probably represent lysosomes (Fig 1A and 1B) During *in vitro* culture for eight days the cell size in both cell populations increased considerably, accompanied by the accumulation of large amounts of phase-dense granules in the perinuclear region (Fig 1C and 1D) The PEC at all stages of culture seemed to be slightly more spread than the monocytes

The survival of PEC in culture was better than that of monocytes After culture for 8 days 73 0 ± 7 4% of the cells present after culture for 105 min were still adherent in PEC cultures as compared to 51 3 ± 9 9% of the monocytes

tion is not clear The aim of this study was to see whether morphological and functional changes similar to those produced in monocytes *in vitro* could be detected in an exudate macrophage population Sterile peritoneal exudate fluid from the indwelling catheter of a patient undergoing peritoneal dialysis was chosen as a source of exudate cells

MATERIALS AND METHODS

Human Peritoneal Macrophages (PEC)

All cells were obtained from the same donor, a 60-year old woman with renal failure due to renal amyloidosis The amyloidosis was secondary to chronic rheumatoid polyarthritis The patient was having regular peritoneal dialysis three times weekly as an outpatient at the time of the experiments Five hundred ml of slightly hypertonic dialysis fluid (1 5% glucose) was left in the abdomen at the end of dialysis sessions The fluid was collected from the permanent indwelling catheter after a period of 60 hs without dialysis just prior to the next session The fluid was collected sterilely in glass bottles with 10 IU heparin/ml Bacteriological tests on each sample were negative The fluid was filtered through sterile gauze to remove gross debris and layered undiluted on cold (4°C) Ficoll Isopaque (Lymphoprep Nyegaard & Co Oslo Norway) After centrifugation at 800 G for 15 min the mononuclear cells were washed twice in Hanks balanced salt solution (HBSS) The cells were suspended in RPMI 1640 (Gibco Bio Cult) Glasgow Scotland) with 20% pooled human A+ serum supplemented by 0 1 mM 1 glutamine and 40 μg gentamycin per ml to a concentration of 2×10^6 cells per ml Aliquots of 250 μl of the cell suspension were applied to circular glass coverslips (Ø 14 mm) placed in dry Petri dishes After incubation for 90 min (5% CO_2 in air 37°C 100% humidity) the coverslips were [illegible] medium and transferred to the

[illegible]

medium was added to each well For the cytostasis assays 0 5 ml of cell suspension with 0.9×10^6 cells per ml was applied directly into the wells of the 24 well plate without coverslips The monolayers were washed once with culture medium and 0 5 ml medium was added after incubation for 90 min The medium was changed after 1 4 and 8 days in wells not previously used for experiments

Human Monocytes

Monocytes were separated from venous blood from healthy adults as described in (11) Briefly blood was defibrinated by a glass stirrer Mononuclear cells were separated by centrifugation on Ficoll Isopaque (800 G for 15 min) The cells were washed three times in HBSS and suspended in culture medium to a concentration of 4×10^6 per ml and aliquots of 0 25 ml were added to coverslips as described for PEC For the cytostasis assay 0 5 ml of the same cell suspension was applied directly into the wells of Linbro plates All furth[illegible] manipulations of the cultures were identical for the tw[illegible] cell populations

Cell Counts

Cells in suspension and Candida particles we[illegible] counted in an electronic particle counter (Coulter mod Fn) Coverslip cultures were incubated for 15 min wi[illegible] 0 5 ml culture medium with 2×10^6 Candida particl[illegible] per ml and washed twelve times in HBSS befo[illegible] counting adherent cells at each point of time The mea[illegible] of 20 visual fields distributed along a full diameter of th[illegible] coverslip multiplied by the number of fields pe[illegible] coverslip was taken as the number of cells on eac[illegible] coverslip

Morphological Studies

Live cells were photographed using a micro chamb[illegible] technique (19) and phase contrast microscope (Lei[illegible] Laborlux) with automatic photographic equipment (Lei[illegible] Orthomat)

Phagocytosis of C albicans

A modification of the assay described by Liken (17) wa[illegible] used Heat killed *C albicans* (Dept of Microbiolog[illegible] University of Trondheim) was labelled with ^{125}I (Kjeller Norway) by electrolysis as described in (16) Th[illegible] specific activity of the labelled Candida particles wa[illegible] determined for each experiment by measuring radioacti[illegible] vity in six replicate samples of 1×10^6 Candida particles and was found to vary between 0 006–0 012 counts pe[illegible] Candida particle

Coverslip cultures of monocytes/PEC were incubate[illegible] for 15 min with 0 5 ml of fresh medium with $2 \times 10^{[illegible]}$ Candida particles per ml after culture for 90 min 1 day 4 days or 8 days The coverslips were washed twelve times in HBSS to remove all extracellular Candida particles and transferred to new wells and 1 0 ml of medium was added Digestion of ingested Candida was allowed to proceed for 24 hours after which the cultures were harvested The medium was removed and centrifu[illegible] ged for 10 min at 2000 G Radioactivity was measured separately in the cell free supernatant the sediment of detached cells and the coverslip with adherent cells in a Packard gamma radiation counter counting lasted for three minutes

The number of Candida particles ingested per cell was calculated as

$$\frac{\dfrac{(\text{cpm coverslip}) + (\text{cpm supernatant}) + (\text{cpm sediment})}{\text{specific activity of Candida}}}{\text{cell number per coverslip}}$$

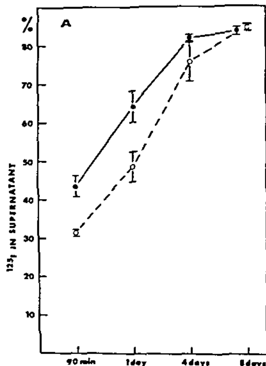
Total cpm per well was 5250 ± 2632 (mean ± SD of all experiments included) Each experiment was performed in quadruplicate

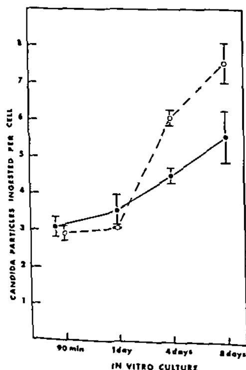
[illegible] reflected [illegible] and was [illegible] calculated as

cells in differentiated macrophage cultures The mean cell density was somewhat higher in PEC cultures This would tend to decrease the PEC phagocytic ability more so in 4 and 8-day cultures because of the better survival of PEC in culture

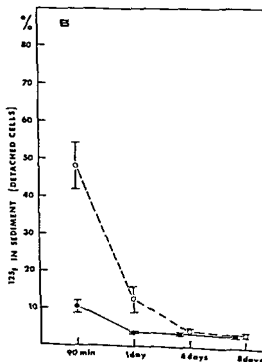
Ab l ty to D gest Ingested C albicans

The PEC had a significantly greater digestion capacity in early culture than the monocytes ($p<0\ 05$ at 90 min and $p<0\ 01$ at 1 day) (Fig 3A) The d gestion capacity of freshly isolated PEC was about the same as that of monocytes cultured for one day *in vitro* During *in vitro* culture the ability to degrade ingested *Cand da* increased to a plateau value where about 85% of the ingested ¹ ⁵I was released during the 24 hour digestion period in 8 day-old cultures The difference between the two cell populations in early culture disappeared both d fferentiating to the same level of maximal d gestion capacity





Fg ² Phagocytic ab l ty of blood monocytes (0—0) or per oneal exudate macrophages (●—●) dur ng *n v tro* d fferen at on Mean ± S E M of exper ments in tr p ca e n 7 for peritoneal exudate macrophages at 90 m n n 6 for per toneal exudate macrophages at 1 day and n 4 for per toneal exudate macrophages at 4 and 8 days n 5 for blood monocytes at all po nts of time



Fg 3 *A* D gest on capacity of blood (0—0) and peritoneal (●—●) mononuclear phagocytes during *in vitro* d fferentiation *B* Cell detachment calculated as % ¹²⁵I in sed ment Symbols as in A C % ¹²⁵I rema n ng

Phagocytic Ability

There was no significant difference ($p>0.5$) between the ability of monocytes and PEC in early culture to ingest ^{125}I labelled *C albicans* (Fig 2) Blood monocytes seem to have a greater capacity for differentiation since they increase their phagocytic capacity 2 6 times during culture for 8 days as compared to a 1 9 times rise in PEC However this may be an effect of different cell densities in the two cell populations since phagocytic ability was found to vary inversely with the cell density in the monolayer being more influenced by the number of

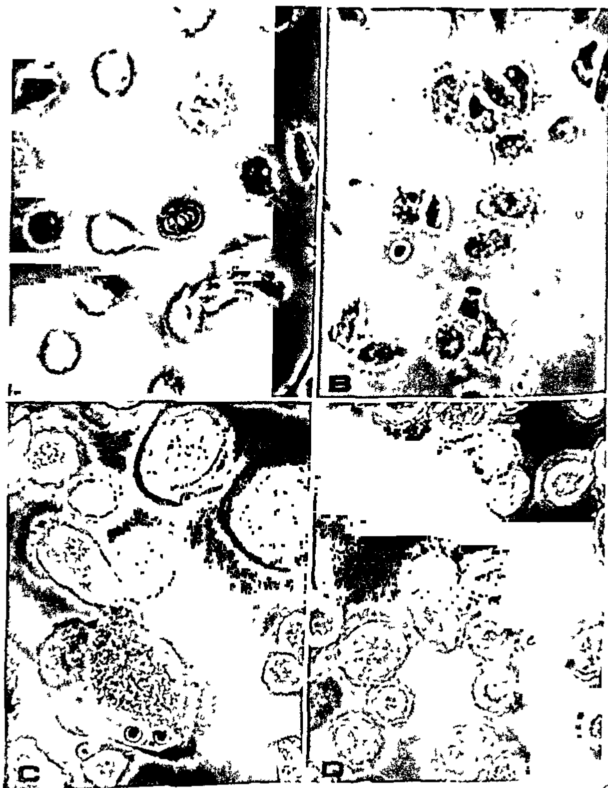


Fig 1 A Peritoneal exudate macrophages after 90 min in culture *B* Blood monocytes after 90 min in culture *C* Peritoneal exudate macrophages after 8 days in culture *D* Blood monocytes after 8 days in culture Phase contrast × 400 Live cells

cells in differentiated macrophage cultures The mean cell density was somewhat higher in PEC cultures This would tend to decrease the PEC phagocytic ability more so in 4 and 8-day cultures because of the better survival of PEC in culture

Ability to Digest Ingested C albicans

The PEC had a significantly greater digestion capacity in early culture than the monocytes ($p<0.05$ at 90 min and $p<0.01$ at 1 day) (Fig 3A) The digestion capacity of freshly isolated PEC was about the same as that of monocytes cultured for one day *in vitro* During *in vitro* culture the ability to degrade ingested *Candida* increased to a plateau value where about 85% of the ingested ^{125}I was released during the 24 hour digestion period in 8 day-old cultures The difference between the two cell populations in early culture disappeared both differentiating to the same level of maximal digestion capacity

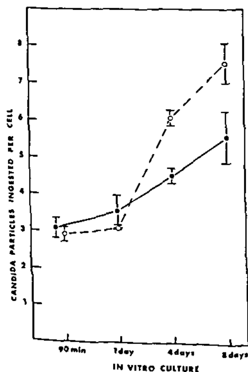
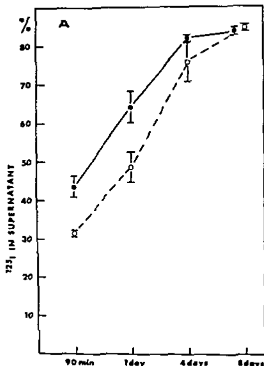


Fig 2 Phagocytic ability of blood monocytes (0—0) or peritoneal exudate macrophages (●—●) during *in vitro* differentiation Mean ± S E M of experiments in triplicate n = 7 for peritoneal exudate macrophages at 90 min n = 6 for peritoneal exudate macrophages at 1 day and n = 4 for peritoneal exudate macrophages at 4 and 8 days n = 5 for blood monocytes at all points of time



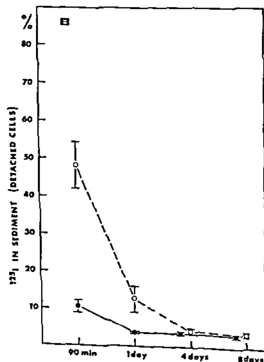


Fig 3 A Digestion capacity of blood (0—0) and peritoneal (●—●) mononuclear phagocytes during *in vitro* differentiation *B* Cell detachment calculated as % ^{125}I in sediment Symbols as in A C % ^{125}I remaining

Phagocytic Ability

There was no significant difference ($p > 0.5$) between the ability of monocytes and PEC in early culture to ingest ^{125}I-labelled *C albicans* (Fig 2) Blood monocytes seem to have a greater capacity for differentiation, since they increase their phagocytic capacity 2 6 times during culture for 8 days as compared to a 1.9 times rise in PEC However, this may be an effect of different cell densities in the two cell populations, since phagocytic ability was found to vary inversely with the cell density in the monolayer, being more influenced by the number o

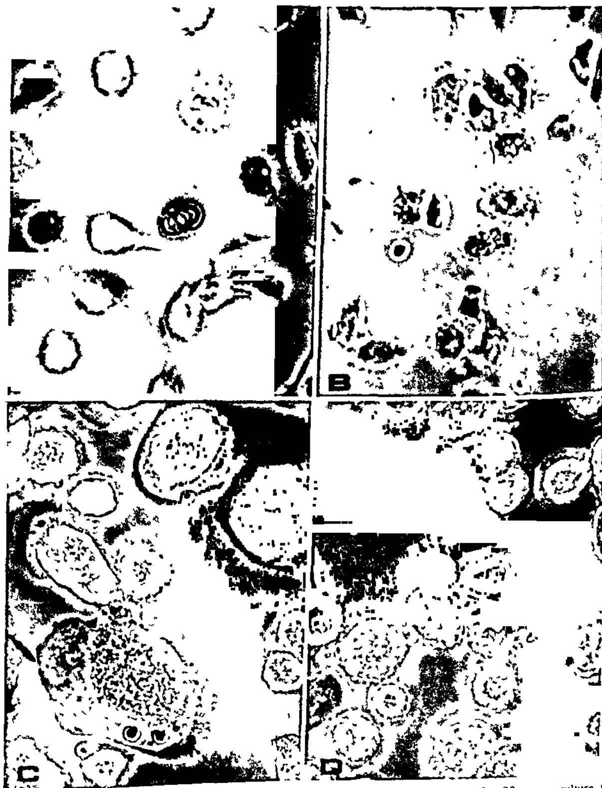


Fig 1 A Peritoneal exudate macrophages after 90 min in culture *B* Blood monocytes after 90 min in culture *C* Peritoneal exudate macrophages after 8 days in culture *D* Blood monocytes after 8 days in culture Phase contrast × 400 Live cells

DISCUSSION

This study demonstrates that human peritoneal exudate macrophages can be isolated relatively easily from patients undergoing peritoneal dialysis thus providing access to a compartment of the mononuclear phagocyte system not previously studied to any great extent in man In animal systems *in vitro* studies on macrophages mainly employ PEC elicited by various chemical or infectious agents and a study of a similar macrophage population in man seemed to be worth while The separation does not cause any inconvenience to the patient

PEC from a patient having peritoneal dialysis can be compared to the more readily available blood monocytes from healthy individuals The obvious comparable monocyte population would of course be the patient s own monocytes but it was found ethically unacceptable to draw the relatively large amounts of blood needed from an anaemic patient with decreased infection resistance The differences observed in effector cell function of PEC and monocytes may however be indicative of relevant functional dissimilarities between the two cell populations and not merely a consequence of PEC donor disease for the following reasons

In our laboratory *Jørstad et al* (3 4) examined blood monocytes from uraemic patients in an assay almost identical with the present study They found that monocytes from [illegible]

[illegible]

adherence to glass after phagocytosis when cultured in medium supplemented by normal serum

The impairment of monocyte phagocytosis digestion and adherence to glass found in uraemic patients (3 4 14) has been shown to be due to factor(s) in uraemic serum (3) All cells in this study were washed and cultured in normal sera thus eliminating this factor as a cause of the difference observed

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cyt

fin

The observation of enhanced effector cell functions in the patient s PEC suggests therefore that this may be due to the difference in anatomical location and *in vivo* differentiation between these cells and blood monocytes

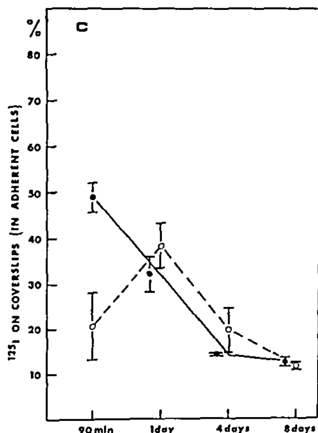
The peritoneal dialysis induces a sterile inflammatory exudate due to the slightly hyperosmolar dialysis fluid Free cells in the peritoneum are probably effectively removed by the dialysis and the exudate fluid sampled 60 hours after dialysis presumably contains largely monocyte-derived macrophages (15) In fact the sampled PEC, having differentiated *in vivo* in the exudate for various periods up to 60 hours resemble *in vitro* differentiated monocytes cultured for 1–2 days as regards the cell functions studied

The data on phagocytosis of *C albicans* does not permit any firm conclusions to be drawn regarding differences in phagocytic capacity, due to the influence of cell density on phagocytosis We could not confirm the finding of *Viken* (17) that phagocytic capacity is constant above a certain cell density Thus comparisons of different phagocyte populations in monolayer culture seem to require very accurate monitoring of the cell density However, our findings suggest that the PEC in early culture are somewhat more active phagocytes at a given cell density Both cell populations increase their phagocytic capacity on *in vitro* culture for 8 days

The degradation of ingested ^{125}I labelled *Candida* with isotope release to the medium is probably a functional assay of crude lysosomal enzyme activity The digestion capacity was significantly higher in the PEC than in the monocytes after 90 min and 1 day *in vitro* thus indicating a more advanced lysosomal function in the *in vivo* differentiated cells The difference becomes indiscernible after one week in culture when both populations reach what is considered to be maximum digestion capacity with complete degradation of ingested Candida

Cell adherence to glass is a membrane function which discriminates sharply between the freshly-isolated phagocyte populations The PEC showed well-developed adherence in early culture which may be interpreted as a sign of »activation« (9) Increased macrophage adherence may however be a differentiation related response Mouse bone marrow macrophages differentiated *in vitro* from non adherent stem cells attach only loosely to plastic after one week in culture (6) The greater adherence of the PEC is matched by monocytes cultured for 1–4 days *in vitro* Thus the greater PEC adherence need not imply »activation« by lymphokines or other mediators *in vivo* but may reflect a differentiation response of the mononuclear phagocyte upon leaving the circulation

The greater cytostatic ability of the PEC in early culture again points to more advanced differentiation in the PEC at isolation as monocytes need more than four days *in vitro* to acquire the same ability to suppress DNA-synthesis in the target cells Cytostatic activity of macrophages *in vitro* has been attributed to supernatant factors (5 10) in some systems affecting ^{3}H Tdr incorporation but not cell proliferation (8) Control experiments with blood monocytes in our system have shown that



in adherent cells on coverslip after the digestion period Symbols as in A Each point represents mean of at least four experiments in quadruplicate for each cell population ± S E M

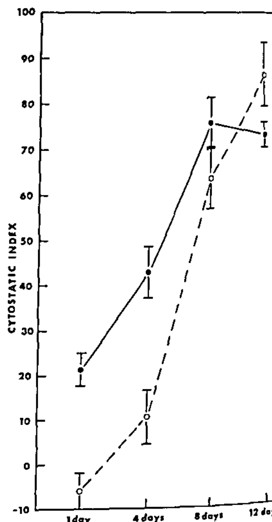


Fig 4 Cytostatic ability of blood (0—0) and perito (●—●) mononuclear phagocytes during *in v* differentiation expressed as cytostatic index Mean ± E M of experiments in triplicate n = 12 for bl cells n = 3 for peritoneal cells

Cell Adherence after Phagocytosis

The ability of the cells to remain adherent to glass after phagocytosis is reflected by the amount of ^{125}I found in the detached cells in the sediment These cells show signs of lysis and death with intact ingested Candida particles PEC in early culture were markedly more adherent than monocytes (Fig 3B) After four days *in vitro* monocytes developed the ability to adhere to glass to about the same degree as PEC after culture for four days This was also verified microscopically and by cell counts made at the beginning and the end of the digestion period

The percentage amount of isotope left on the coverslips after the digestion period (Fig 3C) reflects undigested Candida in viable adherent cells After culture for 90 min the greater adherence of the PEC was reflected by a larger amount of ^{125}I in the adherent cells During the first day of culture the monocyte adhesiveness increased greatly while the rise in digestion capacity was less resulting in higher counts in adherent monocytes on day 1 Thereafter the steady decrease of ^{125}I in adherent cells mainly reflects the improvement in the ability of the cells to degrade ingested fungi into soluble material which is released to the medium

Development of Cytostatic Ability During in v Differentiation

The mononuclear phagocytes were tested their ability to suppress DNA-synthesis in human cell line NHIK 3025 The results are sho in Fig 4

Freshly isolated PEC inhibited ^{3}H-Tdr incor ration in the target cells while freshly isola monocytes exhibited a weak stimulating or »feed effect (0 02 > p > 0 01 for the difference of mo cytes and PEC at day 1) Both cell populations ag displayed an augmentation of effector cell funct on *in vitro* differentiation and matured into mac phages capable of suppressing target cell ^{3}H- incorporation to about 80% of control

Acta path microbiol scand Sect C 87 121–129 1979

A SOLID-PHASE, POLYCLONAL IgM-RF BINDING ASSAY FOR CIRCULATING IMMUNE COMPLEXES

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Institute of Medical Microbiology Odense University DK 5000 Odense Denmark

Glikmann G Svehag S E & Nielsen H A solid phase polyclonal IgM RF binding assay for circulating immune complexes Acta path microbiol scand Sect C *87* 121–129 1979

A polyclonal IgM RF binding assay (pRF BA) for the detection of circulating immune complexes (IC) is described The method is based on the competitive binding of heat treated iodinated IgG (ΔIgG) and naturally occurring IC to solid phase IgM RF The sensitivity limit of the assay was 300–400 ng ΔIgG/ml dilute serum The coefficient of variation for the assay varied from 6 to 12% of the total binding when ΔIgG concentrations up to 1 µg/ml were measured One hundred and six patient sera were examined for IC occurrence and significant differences ($p < 0\ 01$) were observed between 30 normal control sera and sera from SLE sarcoidosis and glomerulonephritis patients About 40% of patients suffering from acute myocardial infarction (AMI) gave IC positive reactions with samples taken 5 to 10 days after the infarction The kinetics of IC appearance was studied in AMI patients by the pRF BA and three complement-dependent assays IC appearance was registered in the RF assay 5 to 12 days after the rise in ASAT enzyme values and the IC reactivity corresponded to complexes ranging from 2 to 5×10^6 in molecular weight

Key words Circulating immune complexes RF binding assay

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Received 15 viii 78 Accepted 31 x 78

Circulating complexes of antibody and antigens of microbial or host origin may deposit in the vascular beds of various organs Such immune complexes (IC) can trigger complement activation and stimulate the release of mediators of inflammatory processes

Several methods for detection of circulating IC have been reported recently Some methods make use of the binding of IC to complement factors [illegible]

[illegible] (21 29) or to rheumatoid factors (4 8 15 16) and conglutinin (5) By use of these methods soluble IC have been detected in patients suffering from a variety of diseases such as rheumatoid arthritis (3 9 17 33 34) chron[illegible]

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This report describes a new competitive protein binding assay in which the binding of solid phase polyclonal IgM rheumatoid factors (RF) to heat aggregated human IgG (ΔIgG) and to soluble IC in pathological sera was measured

MATERIALS AND METHODS

[illegible] was fractionated by gel chromatography on a Sepharose 6B column (Pharmacia Fine Chemicals Uppsala Sweden) equilibrated with Na K phosphate buffer 0 05M pH 7 5 The column was kept at 4° C and the flow rate was 30 ml/hour

The protein concentration of the collected fractions was determined by spectrophotometry and the position of IgM IgG and albumin on the protein elution profile by fused rocket immunoelectrophoresis (26) All fractions were tested for RF activity by the Latex globulin

there is no cytostatic effect of medium conditioned by 12 day old monocytes Medium change and washing of monocytes just prior to the ^{3}H-Tdr pulse does not affect the cytostatic activity It thus seems that the cytostatic ability induced by *in vitro* differentiation of monocytes in human serum is not mediated by stable supernatant factors such as cold thymidine released from monocytes

The indications of increased effector cell function found in freshly isolated PEC suggest that the *in vitro* functional changes induced in monocytes by culture in human serum (13) may be relevant for the *in vitro* differentiation of human mononuclear phagocytes Lodging of mononuclear phagocytes in a sterile exudate did not seem to lead to any restriction of particular functions among those studied The exudate cells showed a capacity to differentiate further *in vitro* into cells structurally and functionally very similar to monocyte derived macrophages This differentiation must be taken into account when studying mononuclear phagocytes *in vitro* as first shown by *Cohn* (2)

The technical assistance of Mrs *M Sorensen* Mrs *B Lippe* and Mrs *A Remen* is gratefully acknowledged I am indebted to Professor *J Lamvik* and Dr *G Unsgaard* for discussion and help and to the Nephrological Section Dept of Medicine Reg Hospital Trondheim for permitting the cell sampling

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This report describes a new competitive protein binding assay in which the binding of solid phase polyclonal IgM rheumatoid factors (RF) to heat aggregated human IgG (ΔIgG) and to soluble IC in pathological sera was measured

MATERIALS AND METHODS

Human Polyclonal IgM RF Preparation

The RF preparation was isolated from sera obtained from a patient with rheumatoid arthritis (RA) Five ml of the serum was fractionated by gel chromatography on a Sepharose 6B column (Pharmacia Fine Chemicals Uppsala Sweden) equilibrated with Na K phosphate buffer 0 05M pH 7 5 The column was kept at 4° C and the flow rate was 30 ml/hour

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that the most suitable test conditions defined as difference between the capacity of IgM RF and IgM NHS to bind the indicator added was obtained when 100 ng of ^{125}I ΔIgG was reacted with 5 μg IgM RF Employing larger amounts of IgM caused an increase in the background counts bound to IgM-NHS coated tubes In addition the results indicated that no further binding of ΔIgG (maximally 11 to 12%) was observed when the tubes were reacted with larger amounts of IgM RF

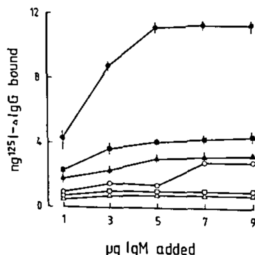


Fig 1 Binding of ^{125}I ΔIgG to solid phase IgM RF (filled symbols) and IgM NHS (open symbols) 50 ng ^{125}I ΔIgG Δ and ▲ 75 ng ^{125}I ΔIgG □ and ■ 100 ng ^{125}I ΔIgG O and ● Range of triplicate determinations (I)

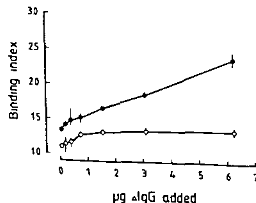


Fig 2 Comparison between competition and inhibition assay Different amo nts of ...

Detection of ΔIgG in the Presence of NHS

A comparison between competition and inhibition assays was made and test conditions were selected based on the findings described above The tubes were coated with 5 μg of the IgM RF preparation as described in Materials and Methods 100 ng of ^{125}I-ΔIgG and different amounts of unlabelled ΔIgG ranging from 9 ng to 6 2 μg were incorporated in 1 ml of NaCl-Tween-BSA solution containing NHS at a final dilution of 1 800 After 3 hours incubation at room temperature the tubes were washed 3 times and the results from the competition assay were recorded In the inhibition assay the solid phase IgM RF tubes were washed with the NaCl-Tween BSA solution and 9 ng to 6

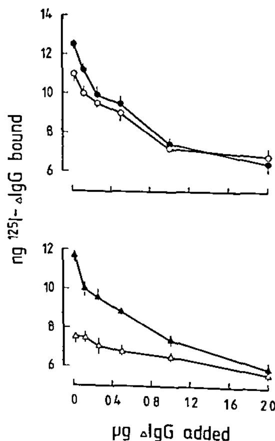


Fig 3 Displacement observed when different amounts unlabelled ΔIgG is incorporated in serum diluted 1 800 in NaCl Tween BSA solution (bottom figure) but not when ser...

agglutination (LA) test Fractions with the highest LA titers in the IgM region were pooled and frozen at −60° C in small aliquots until use The protein concentration of the RF IgM containing pool was 0 6 mg/ml

RF negative normal human sera (NHS) were fractionated similarly by gel chromatography IgM containing fractions from the same region of the elution curve were pooled and stored at −60° C The protein concentration of the control preparation was 0 5 mg/ml

RF determinations

RF activity was demonstrated by latex globulin agglutination (22) and the immunodiffusion method by *Schalén & Christensen* (23) In the agglutination assay *two fold serial dilutions of RA sera* NHS RF positive IgM and RF negative IgM fractions in saline Na tetraborate buffer 0 05M pH 8 5 were added (25 μl) to Takatsy plates and 25 μl of 1 5 diluted latex globulin reagents (Hyland) was introduced into each well The reagents were mixed and the agglutination was recorded after 20 hours incubation at room temperature One latex agglutinating (LA) unit corresponded to about 200 *ng* of the RF IgM preparation

The immunodiffusion assay was performed in 0 6 % agarose using 7 μl of a >22S fraction (10 mg/ml) of a *heat aggregated IgG (Cohn s fraction II)* fractionated on Sephadex G200 in the central well Peripheral wells were filled with 20 μl of undiluted patient sera and the plates were inspected after 2–3 days incubation at 22° C In order to distinguish between RF of IgM and IgG class the sera were tested both untreated and after incubation with 0 15M 2 mercaptoethanol at 22° C for 2 hours

Preparation of [125]I labelled ΔIgG

Aggregated IgG (ΔIgG) was prepared by heating 20 mg human IgG (Cohn s fraction II Kabi Stockholm *Sweden) in 1 ml of a NaCl (0 10 M) Tween 20 (1 %)* BSA (0 2 %) solution at 63° C for 12 min Two ml (10 mg/ml) of the aggregated material was filtered on a Sephadex G 200 column (2 6 × 100 cm Pharmacia Uppsala Sweden) equilibrated with 0 01 M Tris HCl buffer containing 0 5 M NaCl and 0 02 % NaN_3 pH 8 0 The column was run at 4° C flow rate 5 ml/hour

The fractions in the first peak (≥ 22S) were pooled and concentrated to 10 mg/ml Iodination of the heat aggregated material was performed by the lactoperoxidase method (31) The labelled preparations (10–15 *μCi/μg protein*) were separated from free iodide on a Sephadex G 25 column and stored at −60° C

Solid phase RF binding Assay

Five μg of the IgM RF *solution* was added to polystyrene tubes (11 × 70 mm Nunc Denmark) containing 1 ml 0 15 M NaCl buffered with 0 01 M sodium phosphate pH 7 2 (PBS) and the tubes were incubated in a vertical position on a shaker *for 20 hours* at room temperature Controls included tubes incubated with 5 μg of the control IgM preparation and tubes incubated with PBS only

After three washings with NaCl (0 10 M)-Tween *20* (1 %) BSA (0 2 %) − solution triplicate 20 μl test samples diluted 1 8 in the NaCl Tween BSA solution plus 100 to 150 ng of [125]I ΔIgG were added to the coated tubes in 1 ml NaCl Tween BSA The tubes were *incubated further for 3 hours at room temperature* and unbound proteins and labelled reagent were removed by washing three times with cold PBS The bound counts were measured in a Biospan Scintillation detector (Nuclear Chicago)

The analytical error of the assay was determined through a period of 8 days in six different tests using a standard preparation of human ΔIgG SD_{b+w} was 20 % of the CPM bound Results obtained with serum samples from patients were expressed by a binding index CPM bound to IgM RF in the presence of normal human *control sera/CPM bound to IgM RF in the presence of* patient sera A binding index of 1 4 (mean + 2SD for the NHS samples) or greater was taken to indicate an IC positive sample Binding of ΔIgG to IgM NHS coated tubes was negligible A PBS BSA (10 mg/ml) solution was substituted for the NaCl Tween BSA solution when serum samples were examined (see also p 123)

Other Assays for Detection of Soluble IC

Some of the patient sera examined with the solid phase RF binding assay (BA) were tested also for IC *activity by a complement consumption* assay (19) and two solid phase C1q binding assays (7 25)

Fused Rocket Immunoelectrophoresis

The method described by *Svendsen* (26) was used Serum fractions were analysed by the use of rabbit antisera to human immunoglobulins and albumin The sera were obtained from Dakopatts Copenhagen Denmark and Behringwerke AG Marburg West Germany

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RESULTS

Binding of [125]I-ΔIgG to IgM-RF Coated Tubes

Fifty 75 and 100 ng of [125]I ΔIgG were added to tubes coated with from 1 to 9 μg IgM RF and to tubes coated with the same amounts of the control IgM preparation The tubes were incubated for *three hours at room temperature* washed with PBS and bound counts were determined Fig 1 shows

that the most suitable test conditions defined as difference between the capacity of IgM RF and IgM NHS to bind the indicator added was obtained when 100 ng of ^{125}I ΔIgG was reacted with 5 μg IgM RF Employing larger amounts of IgM caused an increase in the background counts bound to IgM NHS coated tubes In addition the results indicated that no further binding of ΔIgG (maximally 11 to 12%) was observed when the tubes were reacted with larger amounts of IgM RF

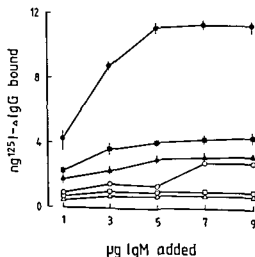


Fig 1 Binding of ^{125}I ΔIgG to solid phase IgM RF (filled symbols) and IgM NHS (open symbols) 50 ng ^{125}I ΔIgG Δ and ▲ 75 ng ^{125}I ΔIgG □ and ■ 100 ng ^{125}I ΔIgG O and ● Range of triplicate determinations (I)

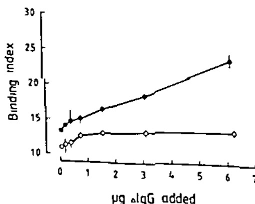


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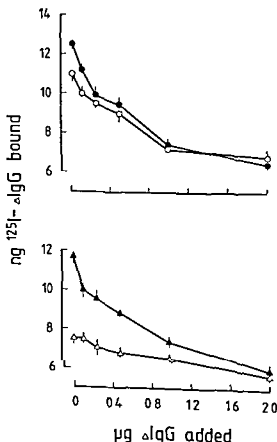


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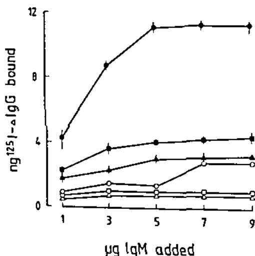


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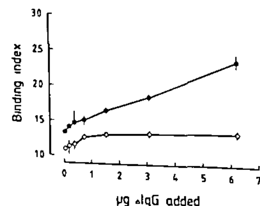
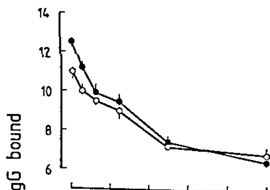


Fig 2 Comparison between competition and inhibition assay Different amounts of inhibitor ΔIgG were tested in the presence of dilute (1 800) normal human serum Inhibition assay (○) competition assay (●) Range of triplicate determinations (I)

Detection of ΔIgG in the Presence of NHS

A comparison between competition and inhibition assays was made and test conditions were selected based on the findings described above The tubes were coated with 5 μg of the IgM RF preparation as described in Materials and Methods 100 ng of ^{125}I ΔIgG and different amounts of unlabelled ΔIgG ranging from 9 ng to 6 2 μg were incorporated in 1 ml of NaCl Tween BSA solution containing NHS at a final dilution of 1 800 After 3 hours incubation at room temperature the tubes were washed 3 times and the results from the competition assay were recorded In the inhibition assay the solid phase IgM RF tubes were washed with the NaCl-Tween BSA solution and 9 ng to 6



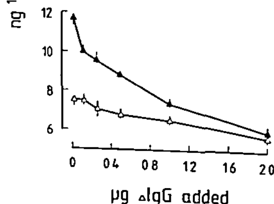


Fig 3 Displacement observed when d fferen
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tions (I)

µg of unlabelled ΔIgG was added to the tubes in the presence of NHS (final dilution 1 800) The tubes were incubated for 3 hours at room temperature, washed three times and 100 ng of ^{125}I-ΔIgG/ml of NaCl-Tween-BSA solution was added After additional incubation for 3 hours, the tubes were washed with PBS three times and the counts bound were determined The results were expressed as a binding index CPM bound in NHS-containing NaCl-Tween-BSA solution/CPM bound in NHS- and ΔIgG-containing NaCl-Tween-BSA solution Fig 2 shows that in the competition assay, within the concentration range of inhibitor ΔIgG studied, a linear relationship between the binding indices and the amounts of ΔIgG added was observed Conversely, in the inhibition assay, significant binding indices (≥ 1 4) were not obtained, even when as much as 6 µg of unlabelled ΔIgG was introduced into the test system (Fig 2) A reduced binding of the indicator ΔIgG was observed when the reaction was performed in the presence of dilute NHS as compared to in the NaCl-Tween-BSA solution This displacement was particularly marked (up to 40% reduction of binding in NHS milieu) when small amounts of inhibitor ΔIgG were used (Fig 3) However, this displacement effect could be reduced strongly by substituting the NaCl Tween-BSA solution with a PBS-BSA solution (10 mg BSA/ml) Using this modification of the assay the degree of indicator binding was not significantly reduced when serum proteins (NHS, 1 800) were introduced into the PBS-BSA solution (Fig 3)

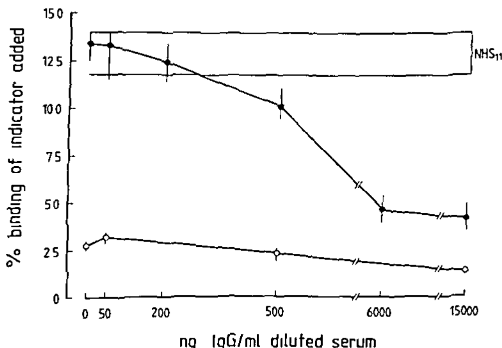
Sensitivity Profile and Reproducibility of the pRF-binding Assay

The sensitivity of the pRF-BA was investigated further as follows 50 ng to 15 µg of ΔIgG was incorporated in NHS (1 800 in NaCl-Tween BSA solution) and added to IgM-RF coated and control IgM tubes respectively A competitive assay was used and the amount of indicator ^{125}I ΔIgG was 100 ng Eleven different NHS were tested

The limit of sensitivity of the test was found to be between 300–400 ng of ΔIgG in a dilute serum milieu (Fig 4) The reproducibility of the assay was evaluated by repeating this experiment thrice in one week Only inhibitor ΔIgG concentrations up to 1 µg/ml could be measured with greater accuracy In this region the analytical error (coefficient of variation) varied from 6 to 12% of the total binding

IC Activity in Sera of Different Patient Groups and Blood Donors

One hundred and six sera from 92 patients with different diagnosed diseases (see Materials and



-- Different amounts of inhibitor ΔIgG were added to NHS indicator ^{125}I-ΔIgG to IgM RF in the presence of inhibitor of inhibitor (O) Binding of indicator to IgM-RF in dilute ted with sera from 11 healthy blood donors (NHS$_{11}$) Range

TABLE 1 *Binding Indices and Latex Agglutination Titers of SLE Sera before and after Absorption Twice to ΔIgG-Sepharose*

Serum	Binding Index*)		Latex aggl titer	
	Before abs	After abs	Before abs	After abs
SLE patient 1	1 74	1 67	1024	32
SLE patient 2	1 96	1 53	512	16

*) Binding index = CPM bound to IgM RF in the presence of NHS / CPM bound to IgM RF in the presence of patient serum

Methods) were tested As controls sera from 20 blood donors were included A binding index (see Materials and Methods) exceeding 1 4 (mean + 2SD for the NHS samples) was taken to indicate IC-positiveness The Wilcoxon test for two samples was used to determine the significance limits for the ranked values of the normal population and each patient group To rule out the presence of free RF in the sera tested which by consumption of indicator ^{125}I ΔIgG could obscure the conclusions made from a competitive assay all sera were screened by the immunodiffusion procedure and examined in the latex globulin agglutination test Sera with demonstrable precipitin reactions and/or with LA titers exceeding the range found for the NHS samples were diluted 1 80 and passed once or, when necessary twice through a small Sepharose ΔIgG column before testing (Table 1) The comparative incidence of positive reactions in the different patient groups is seen in Fig 5 The highest frequency (53 %) of IC-positiveness was observed in sera from patients with glomerulonephritis High binding indices were found particularly in certain sera from SLE patients (Fig 5) A few of these SLE-sera which contained free RF, remained positive when retested after absorption on Sepha-

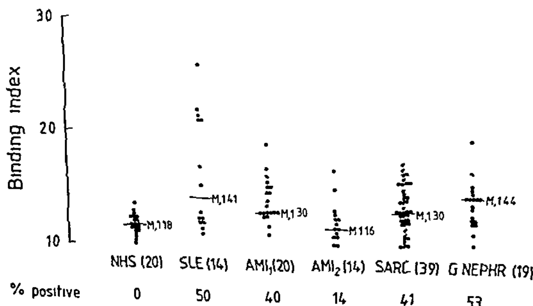


Fig 5 IC reactivity detected by the ...
parentheses after abbreviation ...
(p<0 01) were obtained for the ...
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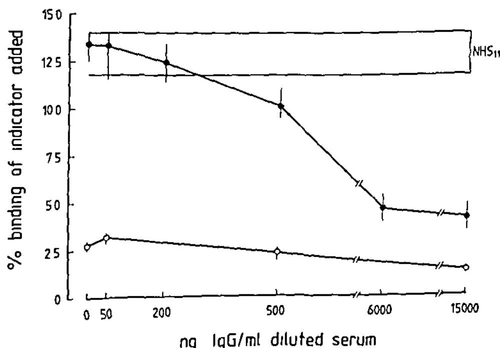
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is presently attempted by using a covalently cross linked ΔIgG preparat on

The solid phase pRF BA has the advantage over the monoclonal RF precipitation technique descri bed by *Winchester et al* (35) of greater sensitivity The threshold of IC detection in the latter is approximately 20 μg/ml while the solid phase RF BA could detect 300–400 ng of ΔIgG *Lurhuma et al* (14) described a method in which IC is measured in terms of its inhibitory effect on the RF or C1q agglutination of IgG coated particles The lower detection limit in this assay was 26 μg aggregated IgG/ml when C1q was used and about 140 μg/ml for the assay based on RF The method described by *Cowdery et al* (4) in which soluble IC are detected by their inhibitory effect on the precipitation of aggregated [illegible] IgG RF was reported to detect as little as 125 ng of soluble complexes However this assay is less suitable for routine use as it takes 4 days to perform a single test *Gabriel & Agnello* (8) recently described a relatively simple and sensitive assay based on the inhibition of ^{125}I monoclonal RF binding to IgG Sepharose A distinct advantage of this method is that free polyclonal rheumatoid actors were reported not to interfere significantly with the assay Comparison of results obtained in different laboratories may however be difficult if monoclonal RF of different specificities are used

Recent studies identifying immune complexes by their reaction with C1q have made use of the precipitation of IC bound to ^{125}I C1q (20 37) or solid phase C1q binding assays (7 8 11 25) These methods are capable of detecting as little as 1 μg ΔIgG/ml but one limitation of these assays is that they only measure complexes with complement fixing activity Platelet aggregation on the other hand is a non-complement requiring highly sensi tive assay for demonstration of soluble IC However only few investigators have been able to master this technique which requires fresh platelets for each assay (21)

Test material containing free RF will interfere with the pRF BA by consumption of indicator I ΔIgG but this problem can be circumvented by diluting the samples and passing them through a small column of ΔIgG Sepharose prior to testing (14) (Table 1) The presence of monomeric IgG in the diluted serum did not seem to interfere with the pRF assay As little as 400 ng of aggregated IgG could be detected even in the presence of about 40 times higher concentration (16 μg) of monomeric IgG

The incidence of IC reactivity was studied in different patient groups by means of the pRF BA and significant differences (p<0 01) between the control sera [illegible] ertain patient groups were observed The highest frequency of IC-positive reactions was noted in sera from patients with glomerulonephritis and SLE

The highest binding indices in the pRF BA was obtained with sera from SLE patients In order to rule out the interfering effect of free RF all sera were screened for RF activity and RF positive sera were diluted and passed throuth a small Sepha rose ΔIgG column prior to testing by the pRF BA This treatment reduced the binding indices in the pRF BA but most SLE sera remained IC positive after removal of free RF

About 40% of the serum samples from patients with acute myocardial infarction showed detectable IC activity when taken 5–10 days after infarction while only 14% of the sera collected 35–45 days after the infarction were positive Autoimmune reactions have been suggested to play a pathogenic role in myocardial disease Anti heart tissue antibodies were reported by *Van der Geld* (32) in patients suffering from postmyocardial infarction syn drome Furthermore deposits of immunoglobulin and complement on the surface of the myofibre were found in biopsy specimens of chronic myocar dial disease by *Hatle & Melbye* (10) The localization of these deposits suggests an autoimmune reaction to antigens of the fibres but it is not known whether the deposits represent primary or secondary events in the developmen [illegible]

pR [illegible]

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infarctions with an interval of about twenty days demonstrated two peaks of IC activity immediately after the first and the second infarction respectively The first peak coincided with IC reactivity demon strated in the CC test whereas the second peak was detected by the pRF BA and the C1q BA only The differential reactivity in the four tests used for IC detection may be due to differences in complement fixing capacity of IC in IC size and the immunoglo bulin class or subclass primarily involved in IC formation

The skilled technical assistance of Mrs *Elsebeth Thomsen* and *Lise Schrøder* and the excellent secretarial assistance of Miss *Elsebeth Orthmann* is gratefully acknowledged The sarcoidosis sera were kindly supplied by Drs *A Christensen* and *G Pallisgaard* sera from patients with acute myocardial infarction by Dr *B Bl th* and RA sera by Dr *P Helby Pedersen* This work was supported by the *Danish Medical Research Council* (project no 512-7193 and 512-8189) *Ingemann O Buck s Foundation* the *National Foundation for the Prevention of Tuberculosis* the *Foundation for the Advancement of Medical Science* and the *Danish Development Agency (DANIDA)*

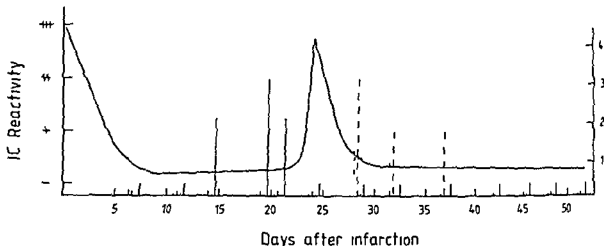


Fig 6 IC reactivity in consecutive serum samples from a hospitalized patient with acute myocardial infarction patient had a second infarction about three weeks after admission to the clinic ASAT enzyme values —— 1 assays for IC detection were used Solid phase pRF BA complement consumption —— solid phase C1q u ^{125}I ΔIgG as test probe – – solid phase C1q BA using ^{125}I protein A as test probe ~

rose ΔIgG columns (Table 1) The median binding index was significantly increased in four patient groups SLE (Med 1 41 p<0 01) sarcoidosis (Med 1 30 p<0 01) glomerulonephritis (Med 1 44 p<0 01) and acute myocardial infarction (serum samples collected 5 to 10 days after infarction Med 1 30 p<0 01) when compared to the normal serum population (Med 1 18)

Kinetics of IC appearence in Patients with Acute Myocardial Infarction

The kinetics of circulating IC appearence in 4 AMI patients was studied by testing consecutive serum samples in the pRF BA a complement consumption (CC) assay (19) and two C1q binding assays (7 25) The temporal relationship between ASAT (aspartate aminotransferase) values and the IC positiveness indicated by the four methods for IC detection is illustrated for one patient in Fig 6

Formation of soluble IC after the first infarction (first sharp increase in ASAT values) was indicated by the RF binding assay and the CC test but not by the C1q binding assays which preferentially detect IC of intermediate and small size A second peak of IC reactivity was recorded in this patient by the C1q binding and RF binding assays at about four weeks after the admittance to the clinic This was unexpected and not observed in 3 other AMI patients which were monitored for IC reactivity by the same tests However subsequently received ASAT values revealed that this patient suffered a second smaller infarction 3 weeks after admission to the clinic

Molecular Si e of Immune Complexes D tecte pRF bindign Assay

Sera from two IC positive patients were fra nated on a Sepharose 2B column using dextran IgM and IgG as markers and eluted fractions monitored by the RF BA in an attempt to est the size of the IC detected IC reactivity observed in the approximate molecular we range from 2 to 5 × 10^{6}

DISCUSSION

Several techniques have been developed for detection of circulating antigen antibody compl in pathological sera Some methods make use of binding of IC to C1q rheumatoid factors conglutinins (4–8 11 14–16 20 24 25) whe others are based on the binding of IC to cell receptors (21 29)

The primary purpose with the present study to develop a rather simple and sensitive RF bin assay for IC determination as a supplement to various C1q and complement-dependent assay current use The solid phase pRF binding as described here is rather easy to perform sufficie sensitive and the analytical error determined testing the inhibitory capacity of unlabelled Δ over a period of one week was conside acceptable The variation within days is prima due to the relative instability of the indic ^{125}I ΔIgG preparations since the RF activity of IgM fraction used remained stable over a perio one year A stabilization of the indicator ^{125}I ΔIgG

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Acta path microbiol scand Sect C 87 131–140 1979

CYTOLOGICAL EVENTS IN ALLO-STIMULATED LYMPHOCYTES TRIGGERED BY EXPOSURE TO STIMULATORY ALLOANTIGENS

I Changes in Cell Size the Mitochondrial Areal Density and Numerical Density of the Endoplasmic Reticulum and the Golgi Apparatus

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Brix Poulsen P Cytological events in allo-stimulated lymphocytes triggered by exposure to stimulatory alloantigens I Changes in cell size the mitochondrial areal density and numerical density of the endoplasmic reticulum and the Golgi apparatus Acta path microbiol scand Sect C 87 131–140 1979

$H\,2^b$ lymphocytes were MLR sensitized against $H\,2^d$ alloantigens and incubated with $H\,2^d$ mastocytoma cells The interactions between lymphoid cells and mastocytoma cells were stopped by fixation with glutaraldehyde Mastocytoma cell lysis was evaluated by the release of ^{51}Cr from ^{51}Cr labelled mastocytoma cells Changes in cell size the amounts of mitochondria endoplasmic reticulum and Golgi apparatus of the lymphocytes were examined and quantitated after 20 and 30 minutes and 1 2 3 and 4 hours of interaction with the mastocytoma cells The stimulated lymphocytes were of two distinct types a small type with a heterochromatic nucleus and few orgenelles (apart from an abundance of free ribosomes) and a larger type with more eucromaci of the nucleus The small lymphocytes showed no increase in cell size or changes in organelle fractions during interaction with the mastocytoma cells The larger lymphocytes increased in diameter during the first 3 hours of interaction with the mastocytoma cells Within the first hour of interaction the large lymphocytes exhibited interdigitations with the plasma membrane of the mastocytoma cells The mitochondrial areal density of the large lymphocytes increased during the first 3 hours of interaction The numerical density of endoplasmic reticulum in the large lymphocytes increased during the first 3 hours interaction whereas the actual concentration was highest at the beginning and decreased until 2 hours after which it increased The concentration of the Golgi apparatus was also highest at the beginning The number of Golgi cisternae per lymphocyte increased during the first 20 minutes and after a delay increased again from 2 to 3 hours of interaction An increase in the total number of Golgi stacks however first appeared after 30 minutes of interaction *Terms used* Areal density = area in percent of cytoplasmic area Numerical density – numbers on cytoplasmic sections

Key words Lymphoid cells lysis mitochondria endoplasmic reticulum Golgi apparatus

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Using electrophoretically separated T and B-cells from mixed lymphocyte culture reaction (MLR) stimulated mouse lymphocytes *Andersson et al* (1973) found that T-cells alone respond in the MLR Mouse lymphocytes depleted of T-cells by treatment with anti θ antiserum and complement do not respond to MLR (*Mosier & Cantor* 1971)

Target cell lysis mediated by lymphoid cells from alloimmunized mice proceeds in three phases the first one (adhesion or recognition phase) and the second one (the lethal hit phase) are effector cell

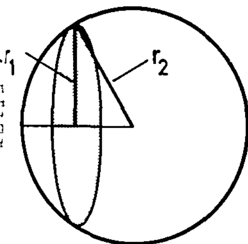


Fig 1 The shaded area indicates the secant plane

The endoplasmic reticulum was quantitated by counting the number of cisternae per μ^2 cytoplasma The Golgi fractions were quantitated by counting the total number of Golgi cisternae the number of stacks per μ^2 cytoplasma and the number of Golgi cisternae per stack

In each observation period 30 to 40 effector cells stimulated against H 2^d alloantigens were counted along with equal numbers of effector cells stimulated against H 2^k alloantigens

Quantitation of ^{51}Cr release The method is described in a pervious article of *Brix Poulsen et al* (1975)

RESULTS

Morphologically distinct cells types The stimulated lymphocytes were morphologically of two distinct types A small type lymphocyte with a heterochromatic nucleus (SH lymphocyte or small heterochromatic lymphocyte) and a large type lymphocyte with more eucromati of the nucleus (LE lymphocyte or large eucromatic lymphocyte)

The SH lymphocyte showed small and few mitochondria an abundance of free ribosomes but only littel endoplasmic reticulum (Fig 2) The LE-lymphocyte showed condensed chromatin only at the nucleolemma and in the form of small islets several mitochondria but fewer free ribosomes and more granulated endoplasmic reticulum than the SH lymphocyte (Fig 2)

The LE lymphocyte concentration was 60 to 80 per cent of the total number of stimulated lymphocytes and remained constant during the four hour observation period

The LE lymphocyte stimulated against $H\text{-}2^k$ alloantigens will in the following be referred to as control lymphocytes

The control lymphocytes and the SH-lymphocytes however, showed no significant increase in cell area or in cell morphology after 4 hours of incubation with the mastocytoma cells

Changes in cell size and morphology At the beginning of interaction with the mastocytoma cells, both types of lymphocytes were present The LE-lymphocyte had a diameter of 5 5 μ, the SH-lymphocyte a diameter of 4 1 μ Table 1 shows LE-, SH-, and control lymphocytes at various intervals during the 4 hour interaction After 3 hours the diameter of the LE-lymphocyte had increased to 13 8 μ or 250 % (Fig 2–4), and the area had increased by approx 450 % (Fig 5) The increasing rate is most pronounced from 0 to 20 minutes after interaction with target cells ($0\ 01 < p < 0\ 02$)

After interaction with the mastocytoma cells for 30 minutes the LE-lymphocytes showed spotlike protrusions and invaginations of the plasma membrane (Fig 6) From 30 minutes to 1 hour of interaction with the mastocytoma cell, interdigitations developed between the plasma membrane of



Fig 2 Stimulated T lymphocytes fixed after 4 days MLR representing the lymphocytes from the beginning of cytolysis a) SH lymphocyte b) LE lymphocyte Magnification 11 570 ×

dependent and can be inhibited by EDTA or heparin (*Martz & Benacerraf* 1973 a & b) the third phase (the phase of target cell disintegration) is effector cell independent (*Golstein & Smith* 1977 and *Martz* 1977) *Berke et al* (1972) found a lag period for initiation of cytolysis of about 10 minutes whereas *Martz* (1976) described a firm adhesion between cytotoxic T-lymphocytes and target cells within 1 minute and 6 minutes later the target cell became »programmed to lyse«

Despite intensive electron microscopic observations the subcellular changes (in immune T-lymphocytes) mediating target cell lysis are still unknown Using PHA stimulated T-lymphocytes *Biberfeld* (1971) observed that lymphocytes usually attach to the target cells by their cytoplasmic pole This pole contains the Golgi apparatus and lysosomal structures *Biberfeld & Johansson* (1975) have shown that there is no focal membrane changes such as deletions membrane fusion or junction type of membrane specialization during the interaction beteeen the lymphocytes and target cells *Liepins et al* (1977) observed interdigitation of the cytoplasma membranes between immunized T-lymphocytes and the appropriate target cells within the first 15 mimutes but found a decrease of surface projections from the lymphocytes after 60 minutes interaction with the target cells

The aim of the persent study was to further examine the cytological changes in immune lymphocytes mediating target cell lysis during the first 4 hours of cytolysis especially alterations in cell size and the amount of mitochondria fraction endoplasmic reticulum and the Golgi apparatus The present observations may provide new knowledge for a better understanding of the initial activation of T lymphocytes mediating target cell lysis

MATERIAL AND METHODS

Animals Mice of inbred strains DBA/2 (H 2^d) C57B1/6J (H 2^b) and C3H/HeJ (H 2^k) were obtained from Gl Bomholtgaard Laven Jutland F_1 hybrids of C57B1/6J × DBA/2J and C57B1/6J × C3H/HeJ were bred at the Institute of Medical Anatomy Copenhagen

Cells *Effector cells* C57B1/6J lymphocytes from ... n and lymph nodes *Stimulator cells* F_1 {C57B1/6J ... d lymph nodes ... × C3H/HeJ} lymphocytes from spleen and lymph nodes *Target cells* The tumour P 815 X2 mastocytoma of the H 2^d origin was kindly donated by Dr *Bent Rubin* Statens Seruminstitut Copenhagen

Cell preparation Spleen and lymph nodes were obtained aseptically and homogenized by grinding through Monodur® PA extra stark 400 The cells were washed once in 4° C phophate buffered saline pH (PBS 420 Gibco) and the cell debris was all we settle Then the cells were spun down and resuspend 156 6 mM NH_4Cl in distilled water at 4° C f minutes disrupting the erythrocytes After three wa the cells were resuspended in culture media and cou

Cultivation medium 500 ml RPMI 1640 25mM Hepes buffer (240 S1 Gibco) containing 25 ml (foetal calf serum (629 Gibco) 5 ml (1 %) L gluta 200 mM (503 Gibco) 125 000 i u Penicillin (N 0 05 g Streptomycinsulfat (Novo) and 5 × 10^{-2} m mercaptoethanol

Stimulation of lymphocytes 30 × 10^6 stimulator or »control stimulator cells« were mixed with ef cells in Falcon tissue culture flask No 3018 with cultivation medium and stored for 4 days at 37° C atmosphere containing 5 % CO_2

Cytolysis fixation and embedding Day 4 initiating the stimulation the lymphocyte susper were counted mixed with mastocytoma cells in a lymphocyte mastocytoma cell ratio adjusted to 10^6 cells per ml cultivation medium and shaked

At time zero 20 and 30 mimutes and 1 2 3 hours after mixture the cell suspensions were down at 400 *g* for 6 minutes

The cytolysis was stopped by fixation in glutaraldehyde in 0 1 M phosphate buffer (pH 7 2 C for 12 hours The cells were than washed for 2 minutes in 0 1 M phosphate buffer (pH 8 2) at Post fixation was performed in 2 % OsO_4 in (phosphate buffer (pH 7 2) for 2 hours at 37° C Th were then washed in 0 1 M phosphate buffer (pH 4° C for 10 mimutes and three times for 30 min 4° C in distilled water Subsequently the cells placed in 0 5 % uranyl acetate in distilled water hours at 4° C and finally washed for 20 minutes a in distilled water The cells were then dehydra increasing concentrations of ethanol and embed Epon 812

Electron microscopy Silver to gray thin section cut on a LKB Ultrotome stained with uranyl aceta lead citrate The cells were then examined and graphed using a Philips EM 300 electron microsc an accelerating voltage of 60 kV

Quantitation and statistics The total cellareas o cell and areas of their cytoplasma and mitoch were measured and calculated stereologically with A S M image system Planes were cut through th at various areas The median value of the cell plane is equivalent to the area of the secant plane half a from the center of the cell (Fig 1) and is u calculate the diameter (D) of the cell as follows w = the median cell area value

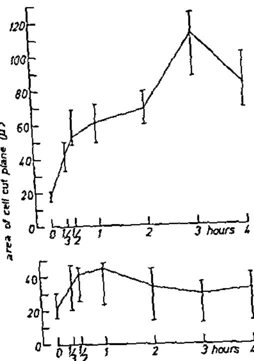


Fig 5 The curves illustrate the areas of secant planes in relation to time of interaction with the target cell The median and the 95% probability for the median are



Fig 6 Large euchromatic lymphocyte (bottom) after half an hour interaction with target cells showing a spotlike protrusion with invaginations (arrows) Magnification 12 500×

he control lymphocytes

Changes in endoplasmic reticulum The LE

< 0.01) after 3 hours of interaction with the mastocytoma cells 67 9% of this increase occurred at 2 to 3 hours of interaction (Fig *10*) However expressed as cisterna per µ² cytoplasma the *endoplasmic reticulum* was *most concentrated* at time zero with 9 38 cisternae per µ² cytoplasma *Fewer cisternae per µ² cytoplasma* would be expected if there was no synthesis while the cytoplasmic volume increased Table 2 column 3 illustrates whether there was synthesis or destruction of cisternae



Fig 7 Interdigitation of the plasma membranes of target cell (T) and the large *euchromatic lymphocyte (L)* after one hour interaction The arrows indicate parts of lymphocyte inside target cell Magnification 32 710×

TABLE 1 *The Relationship between Time and Diameter of Lymphocytes during Interaction with Target Cel*

Time of incubation (hours)	Diameter of LE lymphocytes[a] (μ)	Diameter of SH lymphocytes[b] (μ)	Diameter of control lymphocytes (μ)
0	5 5	4 1	5 9
1/3	8 6	4 1	7 7
1/2	9 5	4 9	8 3
1	10 2	5 1	8 6
2	10 8	6 1	7 5
3	13 8	6 3	7 0
4	11 8	5 6	7 4

a) LE-lymphocytes = large, eucromatic lymphocyte
b) SH lymphocytes = small, heterochromatic lymphocyte

the mastocytoma cell and the LE-lymphocyte (Fig 7), leading in rare cases to long, slender protrusions of the LE-lymphocyte into sheaths from the mastocytoma cells (Fig 8)

Changes in the mitochondrial density Within 3 hours interaction with the mastocytoma cells, the LE lymphocytes showed an increase from 3 3% to 7 6% of the mitochondrial density ($p < 0 0$ followed at 4 hours by a pronounced variation the mitochondrial density (Fig 9)

There were no significant changes in the mi chondrial areal density of the control lymphocy or the SH-lymphocytes during the observati period



Fig 3 Large euchromatic lymphocyte after half an hour interaction with target cells Magnification 7 650 ×



Fig 4 Large euchromatic lymphocyte after 3 hours interaction with target cells Magnification 5 540 ×

TABLE 2 *Change in Number of Endeplasmic Reticulum Cisternae per μ^2 of Cytoplasmic Area in LE lymphocyte in Relation to Time of Interaction with Target Cell*

Time of cytolysis (hours)	LE lymphocyte[a] No/μ^2	Control cells No/μ^2	Cisternae Ratio[b] In LE lymphocyte No/No
0	9 38	9 62	
1/3	4 46	4 78	1 87
1/2	4 39	3 27	1 33
1	3 61	3 02	1 23
2	4 03	4 57	1 20
3	4 79	3 63	1 81
4	4 91	3 03	0 54

[a] LE lymphocyte abbreviation of large eucromatic lymphocyte
[b] The number of cisternae per μ^2 cytoplasma of LE lymphocyte over the expected number per μ^2 cytoplasma if there was no formation and destruction of cisternae from the preceding observation time
> 1 means formation greater than destruction
< 1 means formation smaller than destruction
– 1 means formation equal with destruction

The number of Golgi cisternae per stack increased immediately until 30 minutes decreased until two hours and then increased again during hour two to three of the interaction

Cytotoxicity evaluated with ^{51}Cr release assay

The MLR lymphocytes of $H\ 2^b$ origin stimulated against the mastocytoma cells of $H\ 2^d$ origin generated a specific release of ^{5}Cr from 91 % of the ^{5}Cr labelled mastocytoma cells whereas the control lymphocytes (Stimulated against $H\ 2^k$) only generated a specific release on 22 % (Table 4)

DISCUSSION

It has been shown that in parent – F_1 mixtures of lymphocytes few if any F_1 cells divide despite proliferation of the parental cell (*Wilson 1967 Wilson et al* 1967 *Harrison et al* 1968 and *Clancy & Rieke* 1969) Furthermore in preceding experiments (unpublished) it was shown that after 4 days of stimulation MLR-cultures in which stimulator cells from F_1 hybrids have been treated with mitomycin did not contain fewer blast cells than MLR-cultures in which the stimulator cells were

ABLE 3 *Change in Numerical Density of Stacks and of Golgi Cisternae in LE lymphocyte[a] in Relation to Time of Interaction with Target Cell*

ime of olysis	Change in the stack fraction column 1 No/μ^2	Change in the stack fraction column 2[b] No/No exp	Change in the Golgi cisterna fraction column 3 No/μ^2	Change in the Golgi cisterna fraction column 4[b] No/No exp	Golgi cisternae per stack column 5 No	Stacks per μ^2 cytoplasma of control cells No/μ^2
0	0 144		0 466		3 22	0 157
0 min	0 055	0 94	0 205	1 09	3 75	0 070
0 min	0 028	0 68	0 134	0 80	4 70	0 067
1 hour	0 028	1 22	0 122	0 96	4 39	0 063
2 hours	0 028	1 22	0 099	0 91	3 47	0 057
3 hours	0 025	1 92	0 144	2 36	5 85	0 072
4 hours	0 045	1 09	0 207	1 03	4 65	
						0 069

[a] LE lymphocyte large eucromatic lymphocyte
[b] The num...
there w...
greater ... formatic
... destruction and – 1 indicate formation equal to destructio



Fig 8 Interdigitation of the plasma membranes of target cell (T) and the large euchromatic lymphocyte (L) after 1 hour interaction The arrows indicate protrusions of the lymphocyte in sheaths from the target cells Magnification 14,980 times

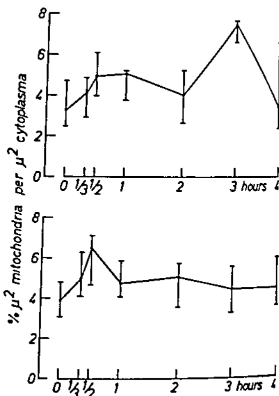


Fig 9 The curves illustrates the areas of mitochondria planes in per cent of the total areas of cytoplasma planes of the LE-lymphocytes (upper graph) and control lymphocytes (lower graph) in relation to time The 95% probability of the median is indicated for each curve

Changes in the Golgi apparatus The concentration of stack per μ² cytoplasma of the LE-lymphocyte, was highest at the beginning of interaction with the mastocytoma cells (Table 3, first column) The number of stacks per LE-lymphocyte decreased during the first 30 minutes of interaction, after which the number of stacks increased Fewer stacks per μ² would be expected if there was no synthesis while the cytoplasmic volume increased Table 3 column 2 illustrates whether there was synthesis or destruction of stacks

The concentration of Golgi cisternae per μ² cytoplasma of LE-lymphocyte was also highest at the beginning of the interaction (Table 3, third column) but the number per cell increased during the first twenty mimutes of interaction From twenty minutes to two hours interaction the number of Golgi cisternae per LE-lymphocyte cytoplasma decreased followed by a profound increase during hour two to three of the interaction Table 3 column 4 illustrates whether there was synthesis or destruction of Golgi cisternae

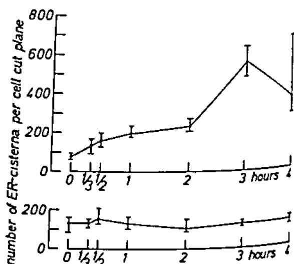


Fig 10 The curves illustrate the numbers of ER-cisterna per cytoplasm planes of the LE-lymphocyte (upper graph) and control lymphocytes (lower graph) in relation to time of interaction with the target cell The median and the 95% probability of the median is indicated for each curve

ABLE 2 *Change in Number of Endeplasmic Reticulum Cisternae per* μ^2 *of Cytoplasmic Area in LE lymphocyte in Relation to Time of Interaction with Target Cell*

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a) LE lymphocyte abbreviation of large eucromatic lymphocyte

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hours	0 025	1 92	0 144	2 36	5 85	0 072
hours	0 045	1 09	0 207	1 03	4 65	0 069

LE lymphocyte = large eucromatic lymphocyte

The number of stacks or Golgi cisternae per μ^2 cytoplasma of LE lymphocyte over the expected number per μ^2 cytoplasma there was no formation and destruction, indicate the change due to cell volume changes Values > 1 indicate formatic greater than destruction < 1 indicate formation smaller than destruction and = 1 indicate formation equal to destructio



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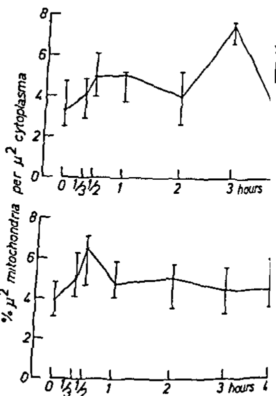


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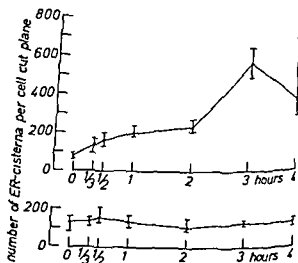


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judged from the observation of ultrastructural changes From the third to the fourth hour of interaction with target cells only small changes are observed in the concentration of Golgi cisternae as well as of whole stacks in the blast killer cell This observation in connection with the decrease in cell size may indicate a great decay of whole Golgi stacks

The conclusion must be that the small blast cell on the fourth day of MLR has a large amount of endoplasmic reticulum ready for fast synthesis of extra Golgi cisternae for the first generation of Golgi apparatus and the formation of the second Golgi generation after one hour of interaction Furthermore the Golgi readiness of the first generation which is increasing from the onset of interaction decreases quickly after twenty minutes

The readiness of the endoplasmic reticulum and the first generation of the Golgi apparatus in the blast cell at the onset of interaction with target cells may explain the observation by *Berke et al* (1972) who found a lag pariod of only ten minutes for the initiation of target cell lysis It has also been shown by *Mart*· (1976) that an electrolyte permeable lesion is produced in the target cell membrane within minutes following contact with the cytolytic T lymphocytes The cytolysis of target cells produced by the cytolytic T lymphocyte is divided in three phases The recognition (adhesion) phase the lethal hit phase and the phase of target cell disintegration (*Golstein & Smith* 1977)

For specific recognition and adhesion in the first phase receptors at the surface of the T lymphocyte is necessary An increase in intracellular transport from the Golgi apparatus of membrane materials to the cell membrane might provide not only an increase in cell size but also an increase in specific structures on the cell surface necessary for the recognition and adhesion The first Golgi generation may be responsible for the intracellular transport of materials to the cell membrane in this first phase

The mitochondrial areal density varies somewhat during the four hours of interaction The decrease in the areal density from the first to the second hour does [illegible]

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	Ratio lymphocytes per mastocytoma cell	Percentage ^{51}Cr release
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Stimulated against H-2^{d}	10/1	85
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untreated MLR-cultures in which the responder cells were treated with mitomycin did not contain blast cells after 4 days The MLR-stimulation used in this experiment may thus be regarded as a »one-way« stimulation of the parental cells by the F_1 cells

In previous studies by *Hayry et al* (1972) and by *Mac Donald et al* (1975) it has been shown that the killer cell occurs in a blast form between the fourth and the eighth day in MLR This paper describes lymphocytes stimulated in MLR for four days, with two distinct types of lymphocytes being found One cell type small and the other one of a larger size the latter identical with a blast killer cell

PHA stimulation of lymphoid cells to blast cell formation is a non-specific stimulation contrary to the MLR, where the blast cells are specifically directed against one particular alloantigen

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This paper describes the increase in blast killer cell diameter after three hours of interaction with target cells The cell diameter increases from 5 4 μ at the onset to 13 8 μ after three hours of interaction This equals a diameter increase of about 250 percent

The alteration of the plasma membrane is in agreement with the observation by *Biberfeld & Johansson* (1975) using PHA stimulated lymphocytes They never observed membrane fusion between the target cell and the effector lymphocytes The interdigitation of the plasma membrane in Fig 7 and the broad contact zone shown in Fig 8, confirm the requirement of contact suggested by *Golstein et al* (1971) and by *Henney & Bubbers* (1973)

Following the MLR stimulation the blast cells are in a state of readiness Thus the highest concentration of endoplasmic reticulum is found in the relatively small blast cells from the beginning of interaction with target cells In spite of a continuing increase in the amount of endoplasmic reticulum throughout the first three hours of the interaction the simultaneous increase in cell size results in a dilution of the endoplasmic reticulum during the first hours From hour three to four strong breakdown of endoplasmic reticulum takes place resulting in an almost unchanged concentration in spite of a decrease in cell size

The Golgi stacks also show a readiness from the start of interaction because the number of stacks per μ^2 cytoplasma only indicates one third of the initi number of twenty minutes This dilution of stack continues the next ten minutes Then the concentra tion of stacks remains constant from thirty minute to two hours of interaction This is achieved b synthesis since the cell size is simultaneousl increasing Thus, the decrease in the concentratio of stacks is a consequence of the strong increase i cell size from the second to the third hour of th interaction

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The conclusion must be that the small blast cell n the fourth day of MLR has a large amount of ndoplasmic reticulum ready for fast synthesis of xtra Golgi cisternae for the first generation of Golgi apparatus and the formation of the second Golgi generation after one hour of interaction Furthermore the Golgi readiness of the first generation which is increasing from the onset of interaction decreases quickly after twenty minutes

The readiness of the endoplasmic reticulum and the first generation of the Golgi apparatus in the blast cell at the onset of interaction with target cells may explain the observation by *Berke et al* (1972) who found a lag period of only ten minutes for the initiation of target cell lysis It has also been shown by *Martz* (1976) that an electrolyte permeable lesion is produced in the target cell membrane within minutes following contact with the cytolytic T lymphocytes The cytolysis of target cells produced by the cytolytic T lymphocyte is divided in three phases The recognition (adhesion) phase the lethal hit phase and the phase of target cell disintegration (*Golstein & Smith* 1977)

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CYTOTOXICITY TO TUMOUR CELLS INDUCED IN HUMAN MONOCYTES CULTURED *IN VITRO* IN THE PRESENCE OF DIFFERENT SERA

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Unsgaard G Cytotoxicity to tumour cells induced in human monocytes cultured *in vitro* in the presence of different sera Acta path microbiol scand Sect C 87 141–149 1979

Rodent macrophages can be stimulated *in vivo* and *in vitro* to become cytotoxic to neoplastic cells It is shown in the present paper that cytotoxicity to a human tumour cell line is induced in *human monocytes* cultured *in vitro* in the presence of human serum The cytotoxic ability is defined as including cytostatic ability measured as inhibition of ^{3}H thymidine (^{3}H TdR) incorporation in tumour cells and cytocidal ability measured as release of radioactivity from ^{3}H TdR labelled tumour cells Monocytes cultured in medium containing 25 per cent human serum (HS M) developed both a cytostatic and a cytocidal ability When tumour cells were separated from these monocytes by a membrane allowing factor mediated interactions a cytostatic effect was found thus indicating that secretion of soluble factor(s) may be an important mechanism The development of cytotoxic ability in the monocytes was accompanied by development of high capacity for phagocytosis of ^{125}I labelled *Candida albicans* increased protein synthesis in the monocytes and microscopically observed alteration into large well-spread monocytes with accumulation of phase-dense granules in the perinuclear region Culture of monocytes in the presence of bovine sera induced less cytotoxic and phagocytic ability as well as a smaller increase in protein synthesis and less morphological alterations as compared to culture in HS M

Key words monocytes cytotoxicity tumour cells serum human

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The functions and morphology of rodent macrophages may be altered by a variety of non specific stimuli Macrophages from mice treated with intraperitoneal injections of proteose peptone typhoid paratyphoid vaccine killed *Corynebacterium parvum* endotoxine pyran copolymer or polyinosinic polycytidylic acid differ in morphology and functions as compared to resident macrophages from untreated mice (3 9 12 15 16) Mouse macrophages can also be modified *in vitro* by endotoxin (12) ds RNA (1) and culture in medium containing bovine sera (14) The stimulated mouse macrophages are usually larger than the resident macrophages They have increased content of phase dense granules and increased activity of lysosomal enzymes (12 14) The ingestion of opsonized (12) and non-opsonized (14) particles is increased as well as the degradation of antigen antibody complexes (15) The ability to restrict growth of intracellular parasites is not improved to the same degree as the ingestion capacity (14)

Stimulated mouse macrophages display cytotoxicity to neoplastic cells (1 3 7 8 9) which would seem to be of importance in host resistance to neoplasms in mice (4) The aim of this study was to determine whether human monocytes possess the ability to develop cytotoxicity to neoplastic cells Experimental modification *in vivo* of human mononuclear phagocytes is not possible for ethical

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reasons Culture of human blood monocytes in the presence of human serum however brings about morphological differentiation into large well spread cells with accumulation of phase dense granules in the perinuclear region (21) Concomitantly the monocytes show increased phagocytic ability (20) enzyme activity (6) and ability to suppress DNA synthesis in stimulated lymphocytes (19) The results of this study demonstrate that the differentiation brought about by culture in the presence of human serum also induces cytotoxic ability in the monocytes to a human tumour cell line

A further object was to study the influence of different sera on the development of cytotoxic ability in the monocytes and to compare the development of phagocytic and cytotoxic ability

MATERIALS AND METHODS

Monocytes Mononuclear cells were isolated from venous blood of healthy human adults as described previously (19) The cells were suspended in RPMI 1640 (Gibco) supplemented by gentamycin l glutamine and 25 per cent pooled human AB serum (HS M) Aliquots of the cell suspension adjusted to 4 × 10^6 cells per ml were added to culture dishes (Ø 35 mm) or applied to coverslips (10 5 × 22 mm) placed in duplicate in culture dishes Five × 10^6 cells were added per culture dish and 1 2 × 10^6 cells per coverslip The cultures were incubated at 37° C with 7 5 per cent CO in air with 100 per cent humidity After 90 min the non adherent cells were removed and fresh HS M added The adherent cells were cultured *in vitro* for 1–12 days before use in experiments HS M was changed after 1 4 and 8 days In some cultures RPMI 1640 with gentamycin and l glutamine supplemented by 25 per cent foetal calf serum (FCS M) 25 per cent newborn calf serum (NBC M) or 25 per cent calf serum (CS M) was used after the first 24 hours of incubation in HS M

Sera The human AB serum (HS) was prepared at the Blood Bank Trondheim Regional Hospital Foetal calf serum (FCS) and newborn calf serum (NBC) were purchased from Gibco Calf serum (CS) was prepared in our laboratory from blood collected sterilely at a nearby slaughterhouse All sera were stored in frozen state (−20° C) in small samples The sera were usually used in experiments shortly after thawing but were occasionally stored at 4° C for 1–2 days before use

Heat treatment of the sera (56° C for 30 min) before addition to the medium resulted in clumping and increased detachment of the monocytes as has also been reported by others (6) Some sera were absorbed twice by addition to washed human erythrocytes (4 1 30 min at room temperature) to remove heterologous antibodies The erythrocytes were fixed with glutaraldehyde (2) to prevent haemolysis The sera were filtered (Millipore 0 22 µm) before use in experiments comparing different sera

Survival of monocytes was registered by counting attached cells in a Reichert inverted phase contrast microscope (× 400) Ten visual fields were counted in each culture The number of cells per dish was calculated by multiplying the mean number of cells per visual field by the number of visual fields per dish

Phagocytosis Monocytes cultured on coverslips in FCS M NBC M or HS M were tested after eight days in culture One ml HS M containing 2 × 10^6 heat killed ^{125}I labelled *Candida albicans* was added per dish ... After further incubation for 15 min the coverslips were washed 12 times in HS M and placed in new culture dishes after which fresh HS M was added After ... hours the medium was centrifuged (1800 g for 10 min) The radioactivity in the supernatant in the sediment and on the coverslips was registered by a Packard Auto Gamma counter

Cytotoxicity In the cytotoxicity assays the target cells used were the human cell line *NHIK 3025* (13) which was isolated from a carcinoma *in situ* of human cervix in 1967 The cells grow in monolayers The doubling time is about 18 hours when cultured in HS M The stock cells grown in culture flasks (Falcon 75 cm) were replated twice a week The cells were detached by replacing medium in the culture flasks with 5 ml 0 ... per cent trypsin solution (Gibco) and agitating with a pipette 3 min later The single cell suspension was centrifuged and the cells were resuspended in HS-M The culture medium of the monocytes was removed before *NHIK 3025* cells in fresh HS M were added

a) *Cytostatic capacity* Monocytes cultured in FCS-M NBC M CS M or HS M prior to the assay were co-cultured with 10^4 or 10^5 tumour cells in 1 ml fresh HS M for 24 hours The controls were tumour cells cultured alone In some experiments the monocytes were heat killed (60° C for 60 min)

In some experiments *NHIK 3025* cells were cultured in membrane chambers (17) proximate to monocytes to allow humoral interaction between monocytes and tumour cells Membrane chambers (MCs) were formed by gluing polycarbonate membranes (Unipore pore size 0 2 µm) to the bottom of 20 mm high glass rings (outer and inner diameter 29 mm and 26 mm) the bottoms of which were widened to fit exactly into the dishes (Ø 35 mm) Double chambers were formed by mounting MCs on 1 mm high rings (outer and inner diameter 34 5 mm and 31 5 mm) which were placed in dishes with or without monocytes The suitable volume of HS M in the lower chamber was 1 3 ml 10^5 *NHIK 3025* cells suspended in 1 ml HS M were added in the MC

Thymidine incorporation was measured by adding 0 5 µCi ^{3}H TdR (sp act 26 Ci/mmol) per ml four hours prior to harvesting The cells were harvested as described previously (19) Calculation was made of the incorporation of radioactivity in monocyte/tumour cell co-cultures as percentage of the incorporation in tumour cells cultured alone

Incorporation in monocytes was < 3 per cent of incorporation in 10^5 tumour cells cultured alone Therefore in the MC experiments only the tumour cells were harvested

b) *Cytocidal capacity* *NHIK 3025* cells in exponential

growth phase were cultured for 24 hours in HS M containing 1 μCi ³H TdR per ml After washing three times with RPMI 1640 the cells were removed with trypsin and resuspended in HS M and 10^4 cells in aliquots of 3 ml were added to monolayers of differentiated monocytes or to control dishes without monocytes Replicates were harvested every day for five days Culture medium containing detached cells was centrifuged (1800 g for 10 min) The sediment and the adherent cells were harvested with a cell harvester as described previously (19) The radioactivity released to the supernatant expressed as percentage of total radioactivity (c p m supernatant + c p m adherent cells and sediment) was calculated

Assay of protein synthesis One μCi/ml of ³H leucine (sp act. 1 0 Ci/mmol) was added four hours prior to harvesting The cells were removed with distilled water and harvested as described for the assay of DNA synthesis (19)

Morphology 10^5 *NHIK 3025* cells were added to dishes containing coverslips with normal or heat killed monocytes Twenty four hours later the coverslips were inverted on glass slides with two ribbons of double sticky tape to form a micro chamber into which medium was filled (21) The cells were studied in a Leitz Laborlux pahse contrast microscope with automatic equipment for photography

Proliferation estimated by counting cells per colony Normal or heat killed monocytes on coverslips were co cultured with 10^4 *NHIK 3025* per dish for 30 hours The cells originating from each tumour cell plated formed a colony The number of tumour cells per colony was counted using the technique described above

Statistics The values were calculated from the mean of duplicates Each value presented is the mean ± SEM of n experiments The p values were calculated using the Wilcoxon Two sample test

RESULTS

Cytostatic capacity of monocytes cultured in HS M Monocytes cultured in HS M were tested for cytostatic effect on the human cell line *NHIK 3025* One-day-old monocytes had no cytostatic effect 4 day-old monocytes were weakly cytostatic while monocytes allowed to differentiate in HS M for 8 or 12 days had a strong cytostatic capacity (Fig 1) These results were found when the monocyte/tumour cell ratio was 3–4 1 as well as 10 times higher Heat killed monocytes (60° C for 60 min) were weakly cytostatic regardless of differentiation of the monocytes at the time of heat killing

There were no aggregates of differentiated monocytes around the tumour cells (Fig 2) However tumour cells co-cultured with differentiated monocytes showed signs of degeneration Granules and vacuoles appeared in their cytoplasm and within 24 hours they usually retracted their

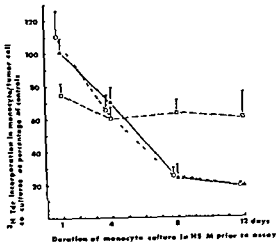


Fig 1 The cytostatic ability to tumour cells of monocytes cultured in HS-M for 1–12 days was tested by adding 10^5 *NHIK 3025* (▲——▲) or 10^4 *NHIK 3025* (O– – –O) per culture Some of the monocyte cultures were heat killed (□ □) prior to addition of 10^5 target cells ³H TdR incorporation was registered during the last 4 hours of the 24 hours assay n = 5

cytoplasmic membrane and tended to detach The tumour cells were not affected by heat killed monocytes in the same way Even though they tended to prefer free areas they grew well on the surface of heat killed monocytes (Fig 3) The heat killed monocytes covered about the same area as live cells

The tumour cell proliferation was evaluated visually by counting *NHIK 3025* cells with a phase contrast microscope The weakness of this method must be considered The results indicated inhibition of proliferation in tumour cells co-cultured with monocytes differentiated in HS M for 8 and 12 days as compared to tumour cells co-cultured with 1 and 4-day-old monocytes (Table 1)

Cytocidal effect of monocytes cultured in HS M Monocytes cultured in HS M for eight days were challenged with 10^4 ³H TdR labelled tumour cells The labelling influenced the growth of the tumour cells Labelled tumour cells cultured alone were unable to proliferate as evaluated microscopically However the diameter of these cells in control cultures increased during the assay period The tumour cells co-cultured with differentiated monocytes seemed to detach shrink and eventually disappear in 3–4 days The differentiated monocytes appeared to be undamaged

The registration of isotope release confirmed the microscopical observation During the first two days the isotope release in the monocyte/tumour




Fig 2 Differentiated monocytes (cultured in HS M for 8 days) were co cultured with *NHIK 3025* cells for 24 hours. The cytoplasmic membrane of the tumour cells is retracted and granules and vacuoles appear in the cytoplasm Phase contrast ×400

Fig 3 Differentiated monocytes (cultured in HS M for 8 days) were heat killed (60° C for 60 min) and then co cultured with *NHIK 3025* cells for 24 hours These tumour cells are like tumour cells cultured alone and grow on the surface of the heat killed monocytes Phase contrast ×400

cell co cultures was at the level of release in controls without monocytes (Fig 4) After three days the release in the former cultures was significantly ($p <$ 0 05) higher than in the control cultures The difference increased during the 4th and 5th days After five days the target cells appeared to be almost completely destroyed by the monocytes

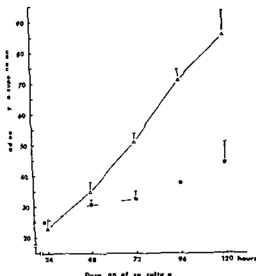
TABLE 1 *Effect of Monocytes Cultured in HS M on Proliferation in NHIK 3025 Cells Co cultured for 30 Hours*

In vitro age of monocytes at the start of the experiments	Proliferation in *NHIK 3025* estimated as number of cells per colony	
	Normal M	Heat killed M
1 day	2 4 ± 0 1	2 4 ± 0 4
4 days	2 5 ± 0 3	2 4 ± 0 5
8 days	1 7 ± 0 3	2 2 ± 0 3
12 days	1 4 ± 0 4	2 3 ± 0 2

Monocytes (M) were cultured in HS M At the start of the experiment 10^4 *NHIK 3025* cells were added per culture dish containing normal or heat killed monocytes After 30 hours the number of *NHIK 3025 cells per* colony was estimated by counting cells in 50 random colonies by phase contrast microscopy n 5

Effect of separating monocytes and tumour cells by the membrane chamber (MC) technique Cell to-cell contact between monocytes and tumour cells was prevented by culturing tumour cells on the polycarbonate membranes of the MCs and monocytes at the bottom of the dishes Monocytes cultured in HS M for 12 days inhibited DNA synthesis in tumour cells to 68 ± 6 per cent of controls (n − 6) No effect was apparent with less differentiated monocytes

Effect of different sera on survival protein synthesis and morphology of monocytes cultured in vitro Monocytes survived both in FCS M and NBC M and in HS M during eight days in culture provided that freshly separated monocytes were incubated in HS M for the first 24 hours (Fig 5) There was no significant increase in ^{3}H leucine incorporation in monocytes cultured in FCS M and NBC M (Fig 6) However the estimated ^{3}H leucine incorporation per cell increased from the 1st to the 4th day as judged by the cell detachment in that period Monocytes cultured in HS M showed a



'g 4 The cytocidal effect of differentiated monocytes cul ured in HS-M for 8 days) was tested by challenging the monocyte monolayer with 10^4 ^{3}H TdR labelled *HK 3025* cells per dish The release of radioactivity to the supernatants during a 5-day period was registered Co-cultures (△—△) controls (O– – –O) n = 6

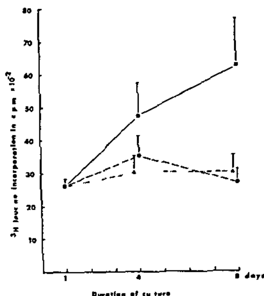


Fig 6 Protein synthesis in monocytes cultured for 24 hours in HS M and for the rest of the period in FCS M (●——●) NBC M (▲– – –▲) HS M (■——■) ^{3}H leucine incorporation was registered by adding isotope (1 µCi/ml) 4 hours prior to harvesting n = 6

significant increase in ^{3}H leucine incorporation both per culture and per cell This is in accordance with the cell morphology The one-day-old monocytes

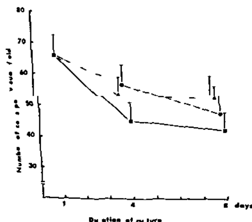


Fig 5 HS NBC cell inverted phase contrast microscope (×400) n 6

were small with retracted cytoplasmic membranes (Fig 7) During the following days the monocytes cultured in HS M were well spread and by eight days the original diameter had increased 3–6 times an a large number of phase-dense granules appeared in the perinuclear region (Fig 8) Monocytes cultured for the same period in FCS M (Fig 9) showed only minor changes as compared to 1-day old monocytes Monocytes cultured in NBC-M and CS M showed a more differentiated morphology but the alterations were less pronounced than in monocytes cultured in HS M

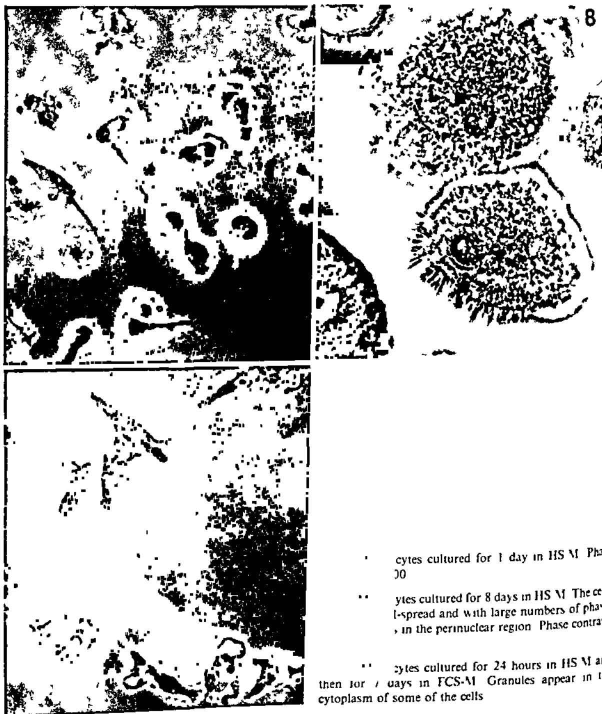
In order to investigate the possible influence of heterologous antibodies the sera were absorbed with human erythrocytes Monocytes cultured in the presence of absorbed sera showed the same morphology as those cultured in unabsorbed sera

Cytostatic ability of monocytes cultured in FCS M NBC M CS M or HS M Monocytes cultured for 24 hours in HS M and then for seven days in FCS M developed a weak cytostatic ability to tumour cells Monocytes ... CS M for ... cytostatic ... M were ... e ector cells in the cytostatic assay (Fig 10)

Phagocytosis of [125]I-labelled Candida albicans *by monocytes cultured in FCS-M, NBC-M or HS-M* Monocytes cultured for eight days according to the protocol described above were tested for phagocytosis of ^{125}I-labelled *Candida albicans* Total radioactivity per culture (c p m in supernatant + c p m in sediment + c p m on coverslips) which measures the ingestion of *Candida albicans* was significantly ($p < 0.01$) higher in the cultures incubated with HS-M prior to phagocytosis than in those incubated with FCS-M or NBC-M (Fig 11a) The percentage of total radioactivity released to supernatants, expressing the capacity of the cells to degrade internalized particles, was also significantly ($p < 0.01$) higher in cells cultured in HS-M than in those cultured in FCS-M and NBC-M (Fig 11b)

DISCUSSION

The results show that human monocytes can be rendered strongly cytotoxic to the human tumour cell line *NHIK 3025* by *in vitro* culture of the monocytes in the presence of human serum The capacity to inhibit growth of tumour cells is largely



cytes cultured for 1 day in HS M Phase
)0

ytes cultured for 8 days in HS M The cells
l-spread and with large numbers of phase
, in the perinuclear region Phase contrast

cytes cultured for 24 hours in HS M and then for 7 days in FCS-M Granules appear in the cytoplasm of some of the cells

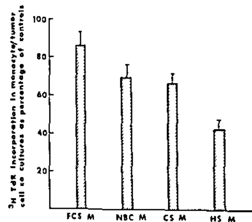


Fig 10 The cytostatic ability of monocytes grown for 24 ho[illegible]

a property of live differentiated monocytes The weakly inhibitory effect of heat killed monocytes is probably not related to the cytostatic capacity of live cells since it is independent of the differentiation of the monocytes prior to heat killing Moreover these results exclude that the cytostatic capacity of live cells is due to reduction of free area available since heat killed effector cells cover about the same area as live cells

The usual monocyte/tumour cell ratio in the cytotoxicity experiments was 3–4 1 The cytostatic effect did not change when 10^4 target cells per culture were added instead of the usual 10^5 This makes malnutrition as cause of the cytostasis unlike[illegible]
depriv[illegible]
ingly[illegible]
malnutrition Furthermore supernatants of culture medium from differentiated monocytes did not inhibit growth in tumour cells

Microscopical studies of prolonged co-cultures of differentiated monocytes and *NHIK 3025* cells revealed that the *NHIK 3025* cells disappeared progressively in 3–5 days This type of delayed tumour cell destruction by *Keller* named cytocidal effect has been observed by using animal macrophages and tumour cells (4 7 8 11) Labelling of tumour cells with ³H TdR makes it possible to follow the monocyte/tumour cell interaction for several days The specific release provoked by differentiated monocytes which is the difference between release in monocyte/tumour cell co cultures and controls (tumour cells alone) was significant from the 3rd day of co-culture in agreement with the morphological observations

The cytocidal effect may be the result of prolonged cytostasis However, the labelling procedure seemed to cause arrest of proliferation as evaluated microscopically This arrest caused no similar destruction Therefore it appears to be unlikely that the destruction is due solely to inhibited proliferation

The cytostatic effect of differentiated monocytes to tumour cells in the MC system indicates that the cytostasis is mediated via humoral factor(s) This

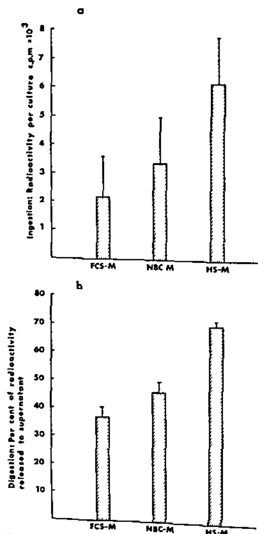


Fig 11 Phagocytosis of 125I labelled *Candida alb cans* by [illegible]
for [illegible]
a [illegible]
b [illegible]
to [illegible]

explanation is not excluded by the fact that monocytes had to be differentiated in HS M for 12 days to acquire cytostatic capacity to *NHIK 3025* cells in the MC system while 8-day-old monocytes were strongly cytostatic in experiments permitting contact with tumour cells Factors working at some distance from the monocytes will be diluted as compared to the concentration close to the mono cytes Furthermore, the factor may be unstable as demonstrated for the monocyte mediated inhibition of proliferation in lymphocytes (17, 18) The latter interpretation may explain the lack of inhibitory effect in supernatants from cultures of 12-day old monocytes

Culture of monocytes in 25 per cent bovine calf sera brought about less change in morphology than culture in 25 per cent human serum This difference was not caused by heterologous antibodies Mono cytes cultured in medium containing foetal calf serum or newborn calf serum showed reduced cytotoxic capacity towards tumour cells reduced capacity for phagocytosis of *Candida albicans* and reduced protein synthesis as compared to monocy tes cultured in medium with human serum Thus bovine sera induce less morphological and functio nal differentiation in the monocytes than human serum This could be due to inactivation of labile components in the commercial bovine sera due to differences in preparation procedures and prolonged storage However this explanation was made unlikely by the fact that freshly prepared calf serum influenced the monocytes in the same way as commercial newborn calf serum

It has been suggested that induction of altered macrophage function is brought about by two types of mechanisms (5 10) an immunological mecha nism involving lymphocyte mediators and a non immunological mechanism involving agents claim ed to have a direct effect on macrophages The importance of the latter type is uncertain because exclusion of lymphocyte involvement is difficult However it seems clear that the morphological and functional changes brought about by culturing monocytes in medium with human serum are independent of lymphocyte products Results not yet published indicate that lymphocyte mediators induce cytotoxic capacity in monocytes by a mechanism which is different from monocyte differentiation

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LYMPHOCYTE BLAST TRANSFORMATION RESPONSE OF SEROPOSITIVE AND SERONEGATIVE SUBJECTS TO HERPES SIMPLEX, RUBELLA, MUMPS AND MEASLES VIRUS ANTIGENS

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Ilonen J Lymphocyte blast transformation respense of seropositive and seronegative subjects to herpes simplex rubella mumps and measles virus antigens Acta path microbiol scand Sect C 87 151–157 1979

Lymphocytes from seronegative and seropositive subjects were stimulated *in vitro* with herpes simplex rubella mumps and measles viral antigens Viral antigens were β propiolactone inactivated crude material (containing cell membrane fragments) grown in Vero cells and prepared identically Lymphocytes from seropositive subjects responded specifically to herpes virus antigen and most rubella and mumps seropositive subjects responded to the respective antigens Measles antigen however did not stimulate lymphocytes from seropositive or seronegative subjects The responses of three subjects studied repeatedly over a period of several weeks were reproducible The results of the study support the usefulness of the blast transformation test as a measure of sensitization to viruses

Key words Lymphocyte blast transformation herpes simplex rubella mumps measles seropositive and seronegative subjects

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The *in vitro* lymphocyte blast transformation response to antigen stimulation is a measure of cell mediated immunity a T lymphocyte function It correlates with other tests of cellular sensitization such as the dermal delayed hypersensitivity and migration inhibition tests (13)

A variety of viral antigens have been found to stimulate human lymphocytes *in vitro* Subjects with disease or vaccination history have responded specifically to herpes simplex vaccinia rubella Epstein Barr varicella and cytomegaloviruses (9 11 21 23 25 27 28 30 31 33 35 40) In some studies various paramyxovirus antigens have also stimulated lymphocytes from patients with multiple sclerosis as well as from adult control individuals (5–7 12 18 34) In other reports however these antigens and especially measles antigens have failed to stimulate lymphocytes (10 26 38) Although there is evidence for the specificity of this stimulation (19) a measles antigen preparation reported to be stimulatory has shown variation among lots

Additional information about the lymphocyte blast transformation test as a measure of cell mediated immunity to viruses was sought by studying responses to identically prepared herpes simplex rubella mumps and measles antigens In addition a highly purified measles antigen preparation was included in the experiments The responses to various antigens were compared with each other and related to the disease history of the subjects as evaluated by virus serology

MATERIALS AND METHODS

Lymphocyte donors Blood samples were collected from patients for minor surgery in a department of paediatric surgery taken at the same time as the samples for routine examination The cells from thirty three child

ren age 7–15 years responded satisfactorily (specific response over 2000 cpm) to at least one of the five virus antigens and purified tuberculin (PPD) used in each experiment PPD was used as a control because all Finnish infants receive BCG vaccination at birth Eight healthy laboratory staff members aged 25–39 years were also studied

Virus antigens All virus antigens were grown in VERO cells maintained in Eagle s basal medium (BME) supplemented with 0 2% bovine serum albumin fraction V 5% tryptose phosphate broth and antibiotics A measles virus strain isolated from a measles patient the VR strain of herpes simplex virus a VERO cell adapted Therien strain of rubella virus obtained from Dr *A Schluederberg* Yale University and a mumps virus strain freshly isolated from a child with acute mumps were used

To prepare the antigens VERO cells were infected with low multiplicity of infection and harvested when the cytopathic effect had extended throughout the cell monolayers The cells were scraped into phosphate buffered saline (PBS) and washed three times with PBS After disruption of the cells by five cycles of freezing and thawing the debris was removed by centrifugation at 2000 *g* for 30 minutes The supernatant was then centrifuged at 80 000 *g* for 30 minutes and resuspended in PBS The antigens were treated with beta propiolactone (BPL) at a final concentration of 0 02% at 4° C for 10 minutes After hydrolysis of the remaining BPL at 37°C for 2 hours and at 4° C for 18 hours the infectivity was tested in VERO cell cultures If all infectivity was not destroyed by the first BPL treatment the procedure was repeated until the preparations were non infectious The quantities of the proteins were determined (20) and the antigens were stored at −20° C in small aliquots until used The control antigens were prepared and stored similarly except that uninfected VERO cells were used The purified measles virus antigen was prepared as described in detail elsewhere (39) The BPL treatment was as described above

Virus antibody determinations The serum specimens were tested for antibodies to herpes simplex parotitis and measles viruses by a microtechnique modification of the complement fixation (CF) method described elsewhere (32) Antibodies to rubella virus were measured by a haemagglutination inhibition (HI) technique (22) The sera with borderline or negative titres were retested in a sensitive radioimmunoassay (1 16 17) to confirm the CF or HI results

Lymphocyte separation and lymphocytic cultures Mononuclear cells were isolated from heparinized peripheral blood by Ficoll Isopaque gradient centrifugation (2) and suspended in RPMI 1640 tissue culture medium Lymphocyte blast transformation experiments were

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12 5 per cent of pooled male serum were distributed in each roundbottomed well of microtitre plates to which 0 05 ml amounts of antigens diluted in phosphate buffered saline were added at the beginning of the incubation For the inhibition test phytohaemagglutinin (PHA P Difco final dilution 1 1000) was added simultaneously with the virus antigens Cultures stimulated with the specific antigens were harvested on day six those with additional PHA on day three ³H thymidine being present for the final 24 hours Median cpm s of triplicate cultures were used to calculate the specific response (cpm of stimulated culture minus cpm of control culture) and stimulation index (cpm of stimulated culture/cpm of control culture)

Statistics The statistical significance for differences between different groups was checked using Wilcoxon s rank sum test

RESULTS

The effect of virus antigens at various concentrations was tested first on lymphocytes from two staff members Both lymphocyte stimulation and the inhibitory effect on PHA stimulation of lymphocytes were studied Final concentration of the antigens were 250 to 0 0025 µg protein/ml in tenfold dilutions except for the purified measles antigen which was tested in concentrations from 25 to 0 0025 µg/ml (Fig 1) The lymphocytes from the herpes seropositive subject (M) responded strongly to herpes antigen Both lymphocyte donors were seropositive for measles rubella and mumps Rubella and mumps antigens each stimulated one of the subjects but measles antigens did not stimulate either one

The highest antigen concentration (250 µg/ml) did not stimulate but inhibited PHA responses markedly herpes antigen inhibited the response by 67 percent rubella antigen by 99 per cent mumps antigen by 27 per cent and (unpurified) measles antigen by 48 per cent Control antigen inhibited only by 2 per cent At a concentration of 25 µg/ml the unpurified antigens inhibited only weakly herpes antigen having the strongest effect inhibiting by 9 per cent At this concentration the purified measles antigen inhibited by 12 per cent In the following experiments concentrations of from 25 to 0 0025 µg/ml of herpes rubella mumps and measles antigens with their control antigen and from 2 5 to 0 0025 µg/ml of the purified measles antigen were used

Specific responses of lymphocytes from 33 children to virus antigens are presented in Fig 2 For each subject and antigen the strongest response induced by the various antigen concentrations is given As a rule this response was induced by a concentration of 25 or 2 5 µg/ml In strong responses the stimulation was seen over a wide range of concentrations The specificity of the response to herpes simplex rubella and mumps viruses was clear the differences between seronegative groups were statistically significant ($p < 0 01$)

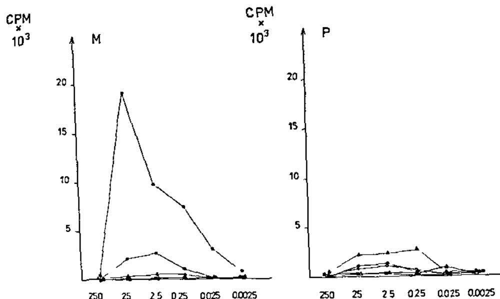


Fig 1 Thymid ne incorporation of lymphocytes from two subjects stimulated with various concentrations of herpes s mplex ● rubella O mumps ▲ and measles △ antigens control •

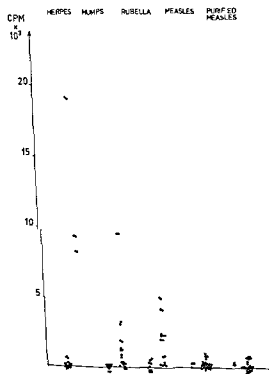


Fig 2 Specif c responses of lymphocytes from seronegative (–) and seropositive (+) children to various virus antigens

Responses to herpes antigen were especially clear with no overlapping between seropositive and seronegative individuals Responses to mumps and rubella virus antigens were usually lower than those obtained with herpes virus antigen Some of the children with mumps and rubella antibodies in their sera did not respond to these virus antigens *in vitro* In seronegative groups none of the children responded discernibly to the relevant virus antigen

Measles antigen prepared in the same way as the other viral antigens did not stimulate lymphocytes in this group of children although only three were seronegative for measles The purified measles antigen slightly stimulated some seropositive subjects

TABLE 1 *Mean and Range of Stimulat on Indices f Lymphocytes from Seronegative ( ) and Seropositive (+) Children to various Virus Antigens*

	seronegative	seropositive
Herpes simplex	1 51 (0 6–5 4)	25 98 (5 0–61 7)
Mumps	1 04 (0 5 1 7)	5 13 (0 7–21 4)
Rubella	1 79 (0 7 3 5)	6 54 (0 6–21 5)
Measles	1 57 (1 0–2 2)	1 36 (0 5– 2 7)
Purified measles	1 77 (1 5–2 3)	2 87 (0 0–19 6)

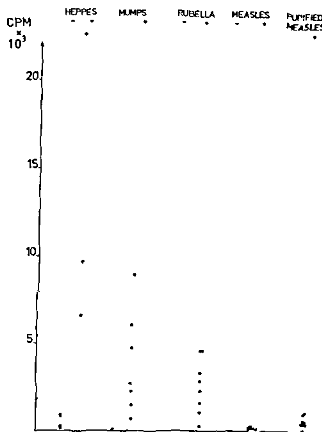


Fig 3 Specific responses of lymphocytes from seronegative (−) and seropositive (+) adult controls to various virus antigens

Responses to various virus antigens are also expressed in Table 1 in terms of mean stimulation index and range Results were essentially similar to those using specific cpm although there was somewhat more overlapping between seronegative and seropositive groups The stimulation index is strongly influenced by the control value indicating spontaneous proliferation and shows great variation with the latter The specific response in cpm describes more reliably the actual strength of stimulation and here also better differentiates the serological groups from each other

In Fig 3, responses to viral antigens of some adult laboratory staff members are shown The responses are quite similar to those of the children

To test the reproducibility of the method three individuals were investigated several times (Fig 4) The responses remained essentially at the same level

DISCUSSION

In this work, similarily prepared herpes simplex rubella, mumps and measles antigens and the lymphocyte blast transformation test were used to investigate cell-mediated sensitization to these viruses The response to herpes simplex virus antigen was strong in all seropositive subjects Rubella and mumps antigens also stimulated specifically lymphocytes from seropositive subjects, but responses were generally lower, and there was a group of seropositive subjects whose cells did not respond to these antigens The identically prepared measles antigen did not stimulate lymphocytes from any of the 33 children and 8 adults who except for three children were all seropositive for measles Highly purified measles antigen elicited low responses in some seropositive children

The inhibitory effect of live measles virus on in

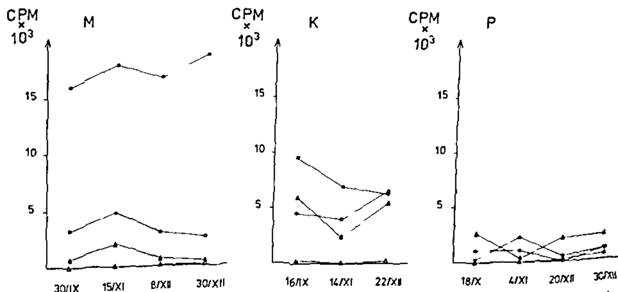


Fig 4 Specific responses of three adult subjects to four virus antigens in experiments at various times Herpes simplex ●, rubella ○ mumps ▲ measles △

ro lymphocyte proliferation is well known (37 41). On the other hand there is much controversy about the effect of various inactivated measles antigens (8, 37, 42, 43). All viral antigens in this study were inhibitory in high concentrations. This phenomenon was not studied further and concentrations used in stimulation experiments did not show inhibition.

The herpes virus is retained in the body after the primary infection and frequent reactivation of the infection is common. This may cause a repeated stimulation of cell mediated immunity which results in strong lymphocyte response to herpes antigen in vitro. Other investigators have also described strong responses to herpes simplex antigens in individuals with herpes history (27, 31, 35).

After rubella and mumps infection cell mediated immunity may subside because of lack of restimulation. After rubella vaccination sensitized lymphocytes have been detected in the blood for only a relatively short time (28, 40) although a natural infection causes a much longer sensitization as measured by the lymphocyte stimulation test in the work of *Rossier and coworkers* (28).

In this study also some adult subjects showed strong responses to mumps and rubella virus antigens many years after childhood infection. *Ma er & So en* (21) found clear responses to rubella virus in most seropositive adults but in their work also some subjects failed to show specific responses in spite of rubella antibodies in their serum. False positive CF reactions to mumps virus because of cross reacting parainfluenza antibodies (4) could also explain the negative blast transformation results of some mumps seropositive subjects.

Some workers have described strong lymphocyte responses to some virus antigens in the absence of specific antibodies (3, 29, 34). They have suggested the lymphocyte transformation test as a more sensitive assay of sensitization than serum antibody measurement. The present work as well as several others (21, 24, 28) does not support that view. To wha e n hor
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In a study of cell mediated immunity in multiple sclerosis the responses obtained with this virus although positive were clearly lower than with other viruses (36). The lack of response to measles antigen in this study may reflect a rapid loss of cellular immunity to measles. Some specific biological features of measles virus may also cause the difficulties in stimulation experiments. The strong response of lymphocytes from a SSPE patient to both purified and unpurified measles antigen (unpublished results from this laboratory) demonstrates however the fundamental ability of these antigens to induce a blast transformation response.

Although cell mediated immunity to the corresponding antigens could not be demonstrated with the *in vitro* blast transformation test in all seropositive subjects the strong responses obtained were highly reproducible. Thus this study supports the usefulness of the blast transformation test as a measure of sensitization to viruses. It needs however to be tested during the course of natural virus infections and in association with vaccinations in order to clarify the formation and persistence of the response as well as individual variations and relations to other immunological phenomenona.

I wish to thank *D A M s onen* for supplying blood specimens from children, Dr *A Salm* for performing antibody determinations and for supplying virus antigens and Mrs *M S nen* for skilful technical assistance.

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CYTOSTATIC EFFECT ON TUMOUR CELLS INDUCED IN HUMAN MONOCYTES BY MEDIATORS FROM BCG-STIMULATED LYMPHOCYTES AND MLC

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Unsgaard G Hammerstrom J & Lamvik J Cytostatic *effect on tumour cells induced in human* monocytes by mediators from BCG stimulated lymphocytes and MLC Acta path microbiol scand Sect. C 87 159–166 1979

Human monocytes activated by mediators (lymphokines) from BCG-stimulated sensitized lymphocytes (from BCG vaccinated donors) were cytostatic to a human cell line Mediators from allogeneic lymphocytes activated the cytostatic ability of monocytes to the same degree as mediators from autologous lymphocytes Mediators from BCG-stimulated lymphocytes from tuberculin negative donors not vaccinated with BCG activated the monocytes only to a small extent. Culture of lymphocytes in a membrane chamber (MC) proximate to monocytes or incubation of monocytes with filtered supernatants of lymphocyte cultures were equally effective procedures for inducing cytostatic ability in monocytes Supernatants of sensitized lymphocytes cultured with BCG for four hours did not activate the monocytes wh le supernatants collected after 24 hours activated the cytostatic ability to the same extent as 72 hour supernatants Supernatants of mixed lymphocyte cultures (MLC) collected after 24 hours d d not activate the monocyte cytostatic ability at all Forty-eight and 72 hour supernatants of MLC showed a small but increasing activity There was no significant difference between BCG st mulated lymphocytes and MLC in their maximum DNA synthesis or in the kinetics of their DNA synthesis Thus the DNA synthesis and secretion of lymphocyte mediators may be independent phenomena resulting from the same stimulus

Key words monocytes lymphokines cytostatic ability BCG MLC

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Mononuclear phagocytes play an essential role in defence against infectious organisms especially intracellular parasites (3 18 20) Accumulating data also indicate that these cells are important effectors in tumour immunity (1 4) Actually there are many similarities between cell mediated antimicrobial immun ty and ...

... macrophages obtained from animals infected with BCG (22) and other intracellular parasites (16 17) are cytotoxic to tumour targets *in vitro* Considerable evidence indicates that both a cytotoxic and a microbicidal effect of macrophages may *be brought about by interaction with lymphocytes* (2 3 9 10 11 12 24 26)

The state of increased microbicidal and tumouricidal capacity of macrophages treated by lymphocyte mediators is usually termed macrophage activation This term has been used to describe changes in several other macrophage abilities such as adherence to glass phagocytosis glucose oxidation through the hexose monophosphate shunt and

RESULTS

Activation of cytostatic ability in monocytes by mediators from BCG-stimulated autologous and allogeneic lymphocytes BCG-stimulated autologous lymphocytes cultured in MCs proximate to monocytes for three days activated the monocytes to inhibit DNA synthesis in NHIK 3025 cells (Table 1) The lymphocytes were stimulated with 1 std BCG per ml (about 10^6 bacilli/ml), which was the optimal dose for induction of DNA synthesis in the lymphocytes (Fig 1) The monocytes activated in this way inhibited the ^{3}H TdR incorporation in the tumour cells to 50 per cent of incorporation in controls (tumour cells cultured alone) Mediators

TABLE 1 *Use of the Membrane Chamber (MC) Technique to Induce Cytostatic Ability in Monocytes by Mediators from BCG Stimulated Lymphocytes*

Culture conditions of monocytes prior to the cytostatic assay	^{3}H-TdR incorporation in monocyte/tumour cell co-cultures c p m $\times 10^{-3}$
MC with non stimulated lymphocytes	164 ± 10
'IC with autologous CG-stimulated lymphocytes	77 ± 14
IC with allogeneic CG-stimulated lymphocytes	85 ± 20[a]
IC with BCG without mphocytes	156 ± 5
o MC	152 ± 13
o MC BCG added to monocytes	147 ± 11
o monocytes umour cells alone	154 ± 7

Monocytes were cultured for 24 hours before MCs were placed in the culture dishes On the 4th day MCs and culture medium were removed and tumour cells were added ^{3}H TdR incorporation was registered from the 20the to the 24th hours of co-culture n = 13 [a]n = 6

B ^{3}H TdR incorporation in replicate lymphocyte cultu res without monocytes Registration on the 4th day

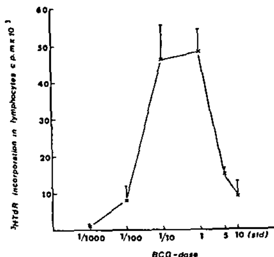
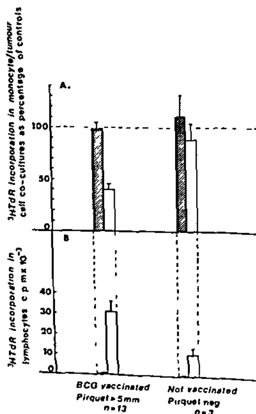


Fig 1 ^{3}H TdR incorporation in lymphocytes cultured in small tubes (10^6 cells in 1 ml HS M per tube) for five days with various doses of BCG One standard unit (std) approx 10^6 bacilli n = 3



activity of different enzymes (5 6 7, 23, 25, 31) However, there is no firm evidence for a link between all these events Actually, there are reports to the contrary which indicate a dissociation between different »activated« functions (33) There fore the term activation will always have to be defined In this article we define it as the altered cytostatic ability induced in monocytes by lympho cyte mediators

In a previous paper it was shown that human monocytes cultured in medium with human serum differentiated into cells with cytotoxic ability to the human cell line NHIK 3025 (28) This modification of monocyte function is probably not influenced by lymphocyte mediators The experiments described in this and the article by *Hammerstrom et al* (13) demonstrate that human monocytes may be rende red cytostatic by lymphokines from lymphocytes stimulated with BCG *Corynebacterium parvum* or allogeneic lymphocytes

MATERIALS AND METHODS

Blood donors Mononuclear leucocytes were isolated from venous blood of healthy BCG vaccinated tubercu lin positive (Pirquet > 5 mm) donors In three experiments the donors were non vaccinated tuberculin negative subjects

Cell cultures Monocytes were isolated and cultured in petri dishes (ø 35 mm) in 1 ml culture volumes as described previously (28 29) The *lymphocyte* population was non adherent cells removed from culture dishes after incubation of mononuclear leucocytes for 90 min Lymphocytes were cultured in a concentration of 10^6 cells per ml Aliquots of 1 ml were cultured in small round bottomed tubes (98 × 16 mm) and aliquots of 4 ml in larger round bottomed tubes (93 × 24 mm) The target cell in the cytostatic assay was the human cell line NHIK 3025 (28) Cells were cultured in RPMI 1640 supple mented with gentamycin 1 glutamine and 25 per cent pooled AB serum (HS M)

BCG One ampoule freeze dried BCG vaccine (Statens Seruminstitut Copenhagen Denmark) was suspended in 2 5 ml Sautons medium A 40 µl sample of this suspension containing about 10^6 bacilli was chosen as one standard unit (1 std) The 1 std suspension was diluted in isotonic saline so that 40 µl contained 1/10 1/100 or 1/1000 std Five std and 10 std were obtained by taking 40 µl and 80 µl respectively of a suspension of one ampoule BCG in 0 5 ml of Sautons medium BCG suspensions were always prepared immediately before use If not otherwise stated the amount of BCG used for stimulation of lymphocytes was 1 std per ml lymphocyte suspension (10^6 cells/ml)

Activation of monocytes by humoral interactions with lymphocytes in membrane chambers (MCs) The MC technique has been described previously (28 30) MCs were placed in culture dishes containing monocytes precultured for 24 hours The MCs were separated from the monocyte monolayer by 1 mm high rings Lympho cytes precultured for 24 hours in small tubes with 1 std BCG or without BCG were transferred to the MCs by pipetting the content of one tube (10^6 cells in 1 ml) into one MC Humoral interactions between lymphocytes and monocytes were allowed to proceed for three days Then the MCs and the culture media were removed and tumour cells were added to the monocyte monolayer in fresh HS M

For registration of DNA synthesis in the lymphocy tes replicates were added to MCs placed in dishes without monocytes

Activation of monocytes by lymphocyte superna[illegible] Lymphocytes cultured for different intervals in tu[illegible] ml per tube) with 1 std BCG per ml or without [illegible] were centrifuged at 800 G for 10 min The superna[illegible] were filtered (Millipore 0 22 µm) and usually [illegible] supernatant was added to the 1 ml culture med[illegible] each monocyte culture (dilution 1 2) In some ex[illegible] ments the supernatants were prepared from [illegible] lymphocyte cultures consisting of 5×10^5 cells pe[illegible] from either donor On the 4th day of monocyte cu[illegible] all medium was removed and tumour cells were add[illegible] fresh HS M Some cultures were washed three t[illegible] with RPMI 1640 to ascertain that residual lymph[illegible] supernatants did not interfere DNA synthes[illegible] lymphocytes was registered in replicates culture[illegible] small tubes (1 ml per tube)

Survival of monocytes on the 4th day in culture [illegible] registered by counting attached cells in an inverted p[illegible] contrast microscope (× 400) Ten visual fields [illegible] counted per culture The number of monocytes per [illegible] was calculated by multiplying the mean number of [illegible] per field by the number of fields per dish The viabilit[illegible] the adherent cells was estimated by counting [illegible] excluding trypan blue (0 4% in 0 15 M NaCl) wh[illegible] was added to the cultures after removal of HS M

Cytostatic ability of monocytes 10^5 tumour [illegible] suspended in 1 ml HS M were added per monoc[illegible] culture making the mean monocyte tumour cell ra[illegible] 2 1 The cells were co-cultured for 24 hours Four hou[illegible] prior to harvesting 0 5 µCi ^{3}H thymidine (^{3}H TdR) ([illegible] act 26 Ci/mmol) per ml was added The results w[illegible] expressed as ^{3}H TdR incorporation in monocyte/tumo[illegible] cell co cultures calculated as percentage of ^{3}H Td[illegible] incorporation in tumour cells cultured alone Incorpor[illegible] tion in monocytes was not subtracted because it ne[illegible] exceeded 3 per cent of incorporation in controls (tumo[illegible] cells cultured alone)

DNA synthesis in lymphocytes was registered b[illegible] adding 0 5 µCi ^{3}H TdR per ml HS M four hours prior [illegible] harvesting Lymphocytes in MCs were transferred [illegible] tubes by washing the MCs vigorously with 5 ml co[illegible] isotonic saline (30)

Harvesting The cells transferred to tubes and the cell[illegible] cultured in tubes during the whole period were harveste[illegible] with a Titertec multiple cell harvester (30)

Statistics The values were calculated from the mean[illegible] of duplicates Each value presented is mean ± SEM of n experiments The p values were calculated using the Wilcoxon two sample test

RESULTS

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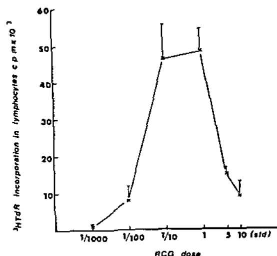


Fig 1 ^{3}H TdR incorporation in lymphocytes cultured in small tubes (10^6 cells in 1 ml HS M per tube) for five days with various doses of BCG One standard unit (std) approx 10^6 bacilli n – 3

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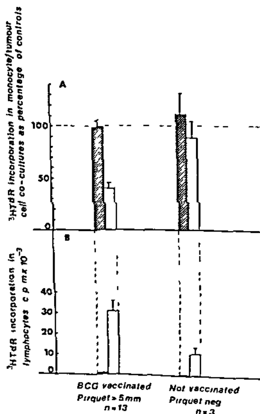


Fig 2 A The ... [illegible] ...cytes from BCG vaccinated or non vaccinated donors On the 4th day MCs and culture medium were removed and NHIK 3025 cells in fresh HS M were added

B ^{3}H TdR incorporation in replicate lymphocyte cultures without monocytes Registration on the 4th day

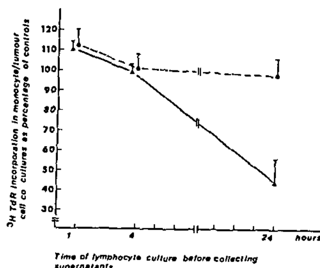


Fig 3 The cytostatic ability of monocytes incubated from the 1st to the 4th day with supernatants from non stimulated lymphocytes (● ●) or BCG stimulated lymphocytes (▲—▲) cultured for 1 4 or 24 hours The supernatants were diluted 1 2 in fresh HS M n = 3

from allogeneic lymphocytes seemed to be as effective as mediators from autologous lymphocytes (Table 1) Non stimulated lymphocytes induced no cytostatic ability in the monocytes Neither did BCG cultured alone in the MC (1std BCG/ml) nor the same dose of BCG added directly to the one day old monocytes (Table 1)

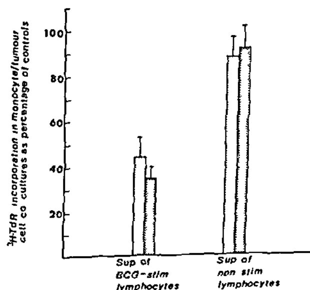


Fig 4 The cytostatic ability of monocytes incubated with supernatants (diluted 1 2 in HS M) form the 1st to the 4th day (▭) or from the 3rd to the 4th day (▨) The supernatants were collected from 24 hour cultures of BCG stimulated or non stimulated lymphocytes n = 6

Donor variations related to BCG vaccination Mediators from BCG stimulated lymphocytes from *non vaccinated tuberculin negative subjects* were only capable to a small extent of rendering autologous monocytes cytostatic (Fig 2) This was shown to be due to lymphocyte properties because the monocytes possessed ability to become cytostatic if exposed to mediators from allogeneic sensitized lymphocytes The reduced ability of the lymphocytes to produce monocyte activating mediators on BCG stimulation was accompanied by a reduced DNA synthesis The latter was registered in replicates in the absence of monocytes

Activation of cytostatic ability in monocytes by supernatants of BCG stimulated lymphocytes Supernatants at optimal conditions were as good activators of cytostatic ability in monocytes as the continuous interaction between monocytes and stimulated lymphocytes obtained by using the MC system Monocytes were incubated with a 1 2 dilution of supernatant from the 1st to the 4th day The culture medium was removed and target cells were added to the monocyte monolayer in fresh HS M Supernatants of lymphocytes stimulated with BCG for one hour and four hours did not activate the monocytes while 24 hour supernatants induced a strong cytostatic ability in the monocytes (Fig 3)

Incubation with active supernatants for 24 hours instead of three days was sufficient to induce optimal cytostatic ability in the monocytes (Fig 4)

The number of monocytes at the start of the cytostatic assay was not different ($p > 0.1$) in the cultures incubated with supernatants from BCG stimulated and non stimulated lymphocytes (Table 2)

TABLE 2 *Survival of Monocytes Incubated with Supernatants of 24 Hour Lymphocyte Cultures*

Monocyte culture conditions from the 1st to the 4th day	Number of cells per visual field	Percentage of cells excluding trypan blue
Supernatants of non stimulated lymphocytes	35 ± 6	>98
Supernatants of BCG stimulated lymphocytes	37 ± 4	>98

Monocytes were incubated with supernatants diluted 1 2 in HS M Number and viability of adherent cells were registered on the 4th day n = 5

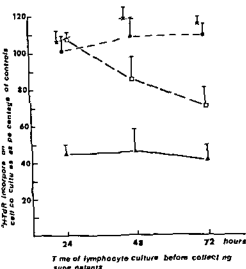


Fig 5 The cytostatic ability of monocytes incubated with supernatants (diluted 1 2 in HS M) from the 3rd to the 4th day The supernatants were collected from BCG stimulated lymphocytes (▲——▲) and non stimulated controls (●   ●) or MLC (□– –□) and non stimulated controls (X   X) cultured for 24 48 and 72 hours n 6

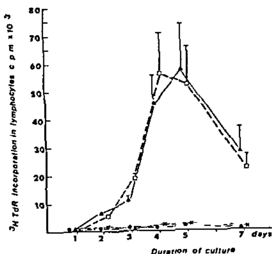


Fig 7 ³H TdR incorporation in BCG stimulated lymphocytes (▲——▲) and non-stimulated controls (●   ●) and in MLC (□– –□) and non stimulated controls (X   X) (10⁶ cells in 1 ml HS M per cultures tube) Replicated were harvested every day during the first five days and on the 7th day n – 4

Activation of monocytes by supernatants from mixed lymphocyte cultures (MLC) compared to activation by supernatants from BCG stimulated lymphocytes Supernatants from MLC cultured for 24 hours did not activate the cytostatic ability in monocytes A small but increasing activity was demonstrated in supernatants from 48 hour and 72 hour cultures (Fig 5) Supernatants from lymphocytes stimulated with BCG for 24 hours were strong activators of cytostatic ability in monocytes No additional effect could be demonstrated by culturing the lymphocytes with BCG up to 72 hours before collecting the supernatants (Fig 5) The DNA synthesis as registered by ³H TdR incorporation in replicates cultured for five days was in MLC

$59 \pm 16 \times 10^3$ c p m and in BCG-stimulated lymphocytes

$54 \pm 9 \times 10^3$ c p m

The monocyte activation by supernatants of 72 hour cultures of BCG-stimulated lymphocytes declined steadily when the supernatants were diluted (Fig 6)

The release of monocyte activating mediators from BCG stimulated lymphocytes preceded the maximal DNA synthesis in the lymphodytes by several days (Fig 7) This was not found for MLC

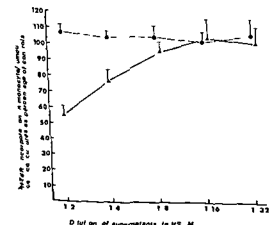


Fig 6 The cytostatic ability of monocytes cultured from the 3rd to the 4th day with various dilutions of lymphocyte supernatants The supernatants were collected from 72 hour cultures of non-stimulated (●   ●) or BCG stimulated (▲——▲) lymphocytes n – 6

DISCUSSION

The results show that BCG-stimulated lymphocytes from BCG vaccinated donors release mediators that render human monocytes strongly cytostatic to the human cell line NHIK 3025 Mediators of both autologous and allogeneic lymphocytes activated the monocytes which is in agreement with results

obtained in rodent systems showing that mediators of even xenogenic lymphocytes activate the cytostatic ability in macrophages (8 9, 10)

Three subjects were not BCG vaccinated but had all been exposed to tuberculin testing When stimulated by BCG the lymphocytes from these donors released less monocyte activating mediators than lymphocytes from vaccinated donors This is in agreement with results in animal systems demonstrating that only lymphocytes from sensitized animals release macrophage activating mediators when stimulated by an antigen (9)

In the only publication known to us to have demonstrated lymphokine activation of tumour cell cytotoxicity in human mononuclear phagocytes the lymphokines were obtained from MLC (12) We found that MLC secreted less monocyte activating mediators than BCG stimulated sensitized lymphocytes even though there was no significant difference in the lymphocyte DNA synthesis following the two types of stimulation There is therefore no proportionality between the secretion of mediators and the intensity of the DNA synthesis The dissociation in the kinetics of mediator secretion and DNA synthesis supports the idea of these being independent phenomena resulting from the same stimulus

The reason why MLC secreted less monocyte activating mediators than BCG stimulated lymphocytes may be that the latter lymphocytes are sensitized to the antigen However *Gougerot et al* (12) found that sensitization did not affect the secretion of macrophage activating factors in human MLC If this is correct our results indicate that the type of stimulation is important for the release of monocyte activating mediators

From our experiments the mechanism for activation of monocytes by BCG in man seems to be mediated solely via lymphocyte products No activation was demonstrated by direct addition of BCG to the monocytes This confirms results obtained in rodent systems indicating that BCG induced activation of several macrophage functions is dependent on lymphocyte mediators (15 34)

injury to the monocytes and release of component from dying monocytes that inhibit growth in NHII 3025 cells because the survival measured a number of adherent cells did not decrease and ther was no increase in the percentage of cells nc excluding trypan blue

Mononuclear phagocytes may influence th production of lymphokines by stimulated lympho cytes Macrophage co operation is necessary fo secretion of mediators by T lymphocytes in re sponse to specific antigens (32) Furthermore humoral factors of activated macrophages have bee demonstrated to cause increased DNA synthesis i stimulated lymphocytes (21) which presumably i accompanied by increased secretion of lymphokines Humoral interaction between monocytes and BCG stimulated lymphocytes proceeding for three day did not however amplify the monocyte activatio beyond the activation promoted by supernatants This is not necessarily in conflict with the paper cited Our lymphocyte suspensions containe enough adherent cells to support antigenic stimula tion (29) Furthermore the monocytes might hav been activated maximally by the supernatants Thirdly, monocytes have been found to inhibi DNA synthesis in stimulated lymphocytes via unstable humoral factor(s) (30) which may also influence the lymphokine secretion

Monocytes are constantly and strongly activated by lymphokines in the *in vitro* system described in this paper The stability of the *in vitro* system and its probable relevance for the *in vivo* situation may make it a useful tool in further investigation of the mechanisms of immunotherapy

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The excellent technical assistance of *M Sorensen B Lippe* and *A Remen* is gratefully acknowledged This work was supported by grants from the *Norwegian Research Council for Science and the Humanities* the *Norwegian Cancer Society* and the *Norwegian Society for Fighting Cancer*

G U is a research fellow of the *Norwegian Research Council for Science and the Humanities* and J H a research fellow of the *Norwegian Cancer Society*

in rat macrophages (27) In order to investigate whether ingestion of BCG may be a possible additional way to activate the cytotoxic ability of monocytes in man a more extensive protocol is needed

The difference between activated and non activated monocyte cultures was not due to an increased number of monocytes in the activated cultures since no increased survival was found in the latter Neither can the results be explained by

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ACTIVATION OF HUMAN MONOCYTES BY MEDIATORS FROM LYMPHOCYTES STIMULATED WITH *CORYNEBACTERIUM PARVUM*

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Hammerstrom J Unsgaard G & Lamvik J Activation of human monocytes by mediators from lymphocytes stimulated with *Corynebacterium parvum* Acta path microbiol scand Sect C 87 167–175 1979

Human monocytes activated *in vitro* by lymphokine-containing supernatants of autologous or allogeneic lymphocytes stimulated *in vitro* by *Corynebacterium parvum* (CP) expressed increased ability to suppress DNA-synthesis in a human tumour cell line Monocyte activation was not dependent on *in vitro* differentiation of monocytes enhanced cytostatic ability being observed at all stages of *in vitro* differentiation The lymphokine induced cytostatic ability was not affected by intensive washing and trypsin treatment of the activated monocytes but disappeared during 48 hours of *in vitro* culture of the activated cells The increased cytostatic ability of lymphokine activated monocytes did not seem to be due to stable supernatant factors released from monocytes CP stimulated DNA synthesis in peripheral blood lymphocytes of 28 normal donors thus confirming the mitogenic effect of CP on human lymphocytes Lymphokine production in response to CP correlated with the magnitude of DNA synthesis but appeared before DNA synthesis could be detected in the lymphocytes

Key words Monocytes human *Corynebacterium parvum* cytostasis lymphocytes lymphokines macrophage activating factor

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The mononuclear phagocyte has been implicated as one of the main effectors of host resistance against neoplasia (10) Previous work with animal systems both *in vitro* and *in vivo* indicate that macrophages acquire the ability to kill tumour cells by activation with mediators (lymphokines) released from stimulated lymphocytes (reviewed in 7) although agents such as *Corynebacterium parvum* (CP) (5) endotoxin (2) and pyran copolymer (31) have been reported to »activate« macrophages without lymphocyte cooperation The term »activation« has been used to denote a wide range of functional changes in lymphokine treated macrophages (discussed in 34) In this study we use it to denote increased cytostatic influence of monocytes induced by lymphokines

The observation that agents effecting macrophage activation also induce tumour regression in animals (23) has led to several immunotherapeutic trials with such agents in man (16 21) One of the main agents employed CP has been analyzed quite extensively in rodents Among the multiple effects produced are antitumour activity (reviewed in 23) increased bone marrow monocyte prod[illegible]
RES
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sion of T-cell mediated immunological reactions (30) and increased resistance to bacterial (1) and viral (18) infections

Results of clinical trials with immunotherapy seem to indicate an urgent need for better understanding of the effects before wide-scale application (8 22) We have only found one study which reports lymphokine activation of human mononuclear phagocytes to tumour cell cytotoxicity (13) and

none indicating whether the effects of CP demonstrated in rodents also operate in man

In this and the article by *Unsgaard et al* (34), we describe results demonstrating that human monocytes can be activated *in vitro* to increased cytostatic influence on human tumour cells by lymphokines CP induces lymphokine release and DNA synthesis in human peripheral blood lymphocytes from normal untreated donors *in vitro* thus indicating that the requirement for *in vivo* presensitization may be different in human and rodent systems

MATERIALS AND METHODS

Human monocytes were separated from venous blood of healthy adults as described in (15) The mononuclear cell suspension obtained from Ficoll/Isopaque centrifugation was adjusted to 4×10^6 cell/ml in RPMI 1640 (Gibco Bio Cult Glasgow Scotland) supplemented with 25% pooled human AB Rh + serum 0 1 mM 1 glutamine and 40 μg gentamycin per ml (HS M) Aliquots of 0 5 ml were incubated in 24 well tissue culture plates (Costar 3524 Costar Cambridge Mass USA well size $17\ 8 \times 16$ mm) for 90 minutes The non adherent cells were aspirated and the monocyte monolayers washed 2–3 times with warm RPMI 1640 This resulted in monolayers of > 90% monocytes determined by phagocytosis of *Candida albicans* with $1–4 \times 10^5$ cells per well determined by visual counting The adherent cell count decreased to about half this value after eight days of culture Medium (0 5 ml HS M) was changed without additional washing after one and four days of culture in wells not used earlier for experiments

Human lymphocytes The non adherent cells aspirated after 90 min incubation of mononuclear blood cells as described above were adjusted to 10^6 cells/ml in HS M and cultured in round bottomed screw capped 16×100 mm glass tubes (Kimax 45066 Kimble Prod Toledo Ohio USA) in aliquots of 1 ml The lymphocyte suspension obtained this way contains about 4% monocytes sufficient to support a normal proliferative response to T cell dependent antigens like PPD and to PHA (33) Tubes were centrifuged at 100 G at the initiation of culture DNA synthesis in lymphocytes was determined by adding 2 μCi of methyl ^3H thymidine (methyl ^3H TdR) (Sp act 5 μCi/mM Radiochemical Centre Amersham England) five hours before harvesting as described in (33) Cultures were assayed in triplicate Results are given as mean counts per minute (cpm) or as net cpm calculated as

(cpm in lymphocytes with CP − cpm in lymphocytes without CP)

The kinetics of lymphokine production were examined by centrifuging the cultures (500 G) after different periods of culture and carefully aspirating the medium which was then discarded One ml of fresh HS M was added and the supernatant harvested as described below after 24 hours of further culture The filtered supernatants were assayed for lymphokine activity on monocytes precultured for three days before supernatant addition as described below

Corynebacterium parvum CP supplied by Dr C *Adlam* Wellcome Research Laboratories Kent, England was cultured and supplied as a freeze-dried preparation by Dr *A Dalen* Dept. of Microbiology University of Trondheim The strain employed was CN 6966 according to the Wellcome classification which corresponds to *Propionobacterium avidum* type I in the *Johnson & Cummins* classification (17) It belongs to the same serological group as the Institute Merieux vaccine IM 1585 (27) and has been shown to posess RES-stimulating activity in mice (unpublished results) Freeze-dried CP was killed by UV irradiation suspended in RPMI 1640 at 1 mg dry weight/ml and stored at −20° C until use

Activation of monocytes with mediators from CP stimulated lymphocytes Lymphocytes were cultured for three days with or without CP 25 μg/ml The tubes were centrifuged (500 G) and the cell free supernatant filtered through 0 22 μ Millipore filters and stored at 4° C if not used immediately No supernatant was stored more than two weeks Addition of CP to control supernatants at harvest before filtering was shown to have no influence on the results and was omitted in most experiments The medium was aspirated from monocyte monolayers and 0 5 ml of supernatants from non stimulated (NS sup) or CP stimulated (CP sup) lymphocytes diluted 1 2 with fresh HS M added After 24 hours the supernatant was aspirated and target cells added as described below

Assay for target cell DNA synthesis The human cell line NHIK 3025 originating from a carcinoma in situ of the cervix (24) was used as target cells Target cell monolayers were trypsinized (0 25% 3 min) and 10^4 cells added to monocyte monolayers in 0 5 ml of fresh HS M resulting in effector target cell ratios of approximately 10 1 DNA synthesis in the target cells was determined by adding 1 μCi methyl ^3H TdR for the last five hours of a 24 hour co-culture period of effector and target cells Cultures were harvested and incorporated radioactivity determined as described in (15) Cultures were assayed in triplicate variation between parallels being less than 10% The results are expressed as % of cpm in target cells cultured in medium alone

$$\frac{\text{cpm (monocytes + NHIK 3025)}}{\text{cpm (NHIK 3025)}} \times 100$$

To describe the activation produced by a supernatant the results are sometimes expressed as cytostatic index (CI) of supernatant treated monocytes relative to monocytes cultured in HS M

$$\text{CI} \quad 100 \quad \frac{\text{cpm(supernatant treated monocytes + NHIK 3025)}}{\text{cpm(untreated monocytes + NHIK 3025)}} \times 100$$

Incorporated radioactivity in monocytes was not subtracted since this did not exceed 5% of cpm in target cells cultured alone Target cell methyl ^3H TdR incorporation in controls without monocytes was 60576 ± 4127 cpm (mean ± SEM of all experiments included)

Statistics P values were obtained by Wilcoxon s two-sample test Results given are mean ± SEM of n experiments

TABLE 1 *Activation of Human Monocytes with Supernatants from CP stimulated Lymphocytes*

Monocyte treatment	Cpm in target cells as % of target cell control			
	Monocyte culture time before addition of supernatant			
	3 days	p	7 days	p
HS-M	103 3 + 3 4		52 9 ± 2 1	
NS sup	93 2 ± 4 0		51 2 ± 5 6	
CP-sup	60 3 ± 3 6	<0 005	17 8 ± 1 5	0 016

Mean ± S E M
n – 32 at 3 days n – 6 at 7 days
p values relative to NS sup

RESULTS

Activation of monocyte-mediated cytostasis by supernatants from CP-stimulated lymphocytes Monolayers of human monocytes incubated with supernatants from CP-stimulated lymphocytes inhibited DNA synthesis in human target cells significantly more than monocytes treated with control superna-

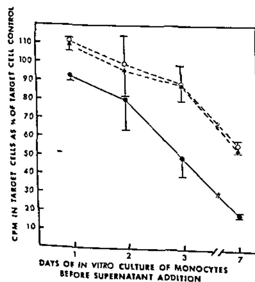


Fig 1 Methyl 3H TdR incorporation (cpm) in NHIK 3025 target cells as % of cpm in NHIK 3025 cultured in medium alone in
▲——▲ HS-M treated monocytes + NHIK 3025
○——○ NS sup treated monocytes + NHIK 3025
●——● CP-sup treated monocytes + NHIK 3025
Mean ± S E M
n = 3 at 1 and 2 days n = 5 at 3 and 7 days

Table 2 *Reversal of Supernatant-induced Activation by in vitro culture of Activated Monocytes*

Time of assay	Cytostatic index
1 Immediately after activation	47 3 ± 10 6
2 Following 48 hours of culture in HS-M	−0 7 ± 2 2

Mean ± S E M
n = 4
Monocytes cultured for 3 days before addition of supernatants from CP-stimulated lymphocytes
1 Target cells added on day 4
2 Fresh HS M added on day 4 target cells added on day 6

tants or HS M only (Table 1) Activation was accomplished at all stages of monocyte *in vitro* differentiation tested (Table 1 and Fig 1) Freshly isolated monocytes, which in this assay actually stimulated target cell prol ferat [illegible]
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Reversal of activation by in vitro *culture of activated monocytes* In order to examine whether the increased cytostatic ability produced by lymphokine supernatants reflected a permanent change in the monocytes supernatant [illegible]

[illegible] induced increase in cytostatic ability, expressed as CI, had disappeared completely by this time, the *activated* monocytes showing the same

TABLE 3 *Lack of Cytotoxicity of Supernatants from Activated Monocytes*

Supernatant source	Cpm in target cells as % of target cell control	
	5 hours exposure to supernatant	29 hours exposure to supernatant
HS M treated monocytes	108 6 ± 2 3	99 2 ± 1 0
CP sup treated monocytes	105 9 ± 6 5	103 0 ± 6 2

Mean ± S E M
n = 5 for 5 hours exposure n = 4 for 29 hours exposure
Monocytes cultured for three days before addition of HS M or supernatants from CP stimulated lymphocytes Fresh HS M added on day 4
Supernatants from HS M or CP sup treated monocytes collected and centrifuged day 5
On day 5 0 25 ml of medium was withdrawn from target cells plated alone on day 4 and 0 25 ml of monocyte supernatant was added One µCi of methyl ³H TdR was added immediately (5 hours exposure) or 24 hours later (29 hours exposure) and the cultures were harvested after 5 hours of methyl ³H TdR incorporation

level of cytostatic ability as HS M-cultured monocytes There were no signs of cytotoxic influence on the supernatant treated monocytes as cell numbers and numbers of cells excluding 0 4 % trypan blue were not different in HS M and CP sup treated monocytes when tested in parallel cultures on day 6 (data not shown)

Lack of cytostatic ability of supernatants from activated monocytes Macrophage mediated cytostasis has in several models been attributed to supernatant factors (9 20 26) To test whether such factors might be responsible for the increased cytostatic ability of activated monocytes supernatants of activated monocytes were added to target cells cultured without monocytes (Table 3) No cytostatic activity in monocyte supernatants could be demonstrated by this procedure

Effect of intensive washing trypsin treatment and freezing and thawing of activated monocytes Control experiments were performed to test the following hypotheses The cytostatic influence might be due to lymphotoxin contamination of the wells or adherent lymphocytes might be mediating the effect Intensive washing or trypsin treatment of the activated monocytes before target cell addition did not however decrease their cytostatic ability significantly (Table 4)

In order to examine whether the activation effect depended on increased levels of toxic molecules accumulating in the activated cells the monolayers

TABLE 4 *Influence of Intensive Washing and Trypsin Treatment of Monocytes Activated with Supernatants from CP stimulated Lymphocytes*

Monocyte treatment	Cytostatic index	p
1 CP sup	47 9 ± 5 1	
2 CP sup + washing	43 6 ± 2 7	n s
3 CP sup + trypsin	51 1 ± 6 9	n s

Mean ± S E M n = 5
Monocytes cultured for 3 days before addition of supernatant
2 Monocytes washed 12 × with RPMI 1640 before addition of target cell on day 4
3 Monocytes washed once with RPMI 1640 exposed to 0 25 % trypsin for 3 min and washed once more before addition of target cells
p relative to CP sup

TABLE 5 *Influence of Freezing and Thawing Supernatant activated Monocytes*

Monocyte treatment	Cpm in target cells as % of target cell control
HS M + freeze/thaw	43 8 ± 8 7
NS sup + freeze/thaw	50 1 ± 11 1
CP sup + freeze/thaw	18 2 ± 4 3

n = 3
Mean ± S E M
Monocytes cultured for 3 days before addition of supernatant
On day 4 the monocytes were washed once and frozen at −20° C for 3 hours After 1 hour of thawing and rewarming at 37° C 100 % humidity target cells were added

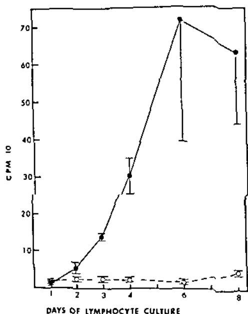


Fig 2 Kinetics of methyl ³H TdR incorporation in CP stimulated lymphocytes
O——O Non stimulated lymphocytes
●——● Lymphocytes cultured with CN 6966 25 µg/ml
Mean ± S E M
n = 5

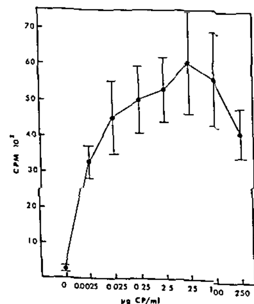


Fig 3 Dose/response curve of methyl ³H TdR incorporation in CP-stimulated lymphocytes cultured for 5 days
Mean ± S E M
n = 5

were washed and the monocytes killed and disrupted by freezing and thawing once before the addition of target cells At the time of the addition of methyl ³H TdR 19 hours later, no intact monocytes could be seen by phase-contrast microscopy Target cells exposed to disrupted activated monocytes were inhibited more than those exposed to medium or control supernatant treated monocytes disrupted in the same way (Table 5)

DNA-synthesis in human lymphocytes stimulated with CP Human peripheral blood lymphocytes responded to killed CP by DNA-synthesis from the second day of culture, with a peak response between 4–8 days (Fig 2) The dose response relationship (Fig 3) shows that CP induces almost maximal responses over a broad range of concentrations The concentration of 25 µg/ml was chosen for experiments with lymphokine production in CP-stimulated lymphocytes All donors tested responded to CP, with a wide variation of responses (Fig 4)

Kinetics of lymphokine production The degree of cytotoxic ability induced in monocytes by a lymphocyte supernatant is presumed to reflect lymphokine concentration, because a dose response relationship can be demonstrated (34) Lymphokine activity, expressed as cytostatic index produced during the last 24 hours of lymphocyte culture at different stages of CP induced lymphocyte stimulation is shown in Fig 5 Almost maximal

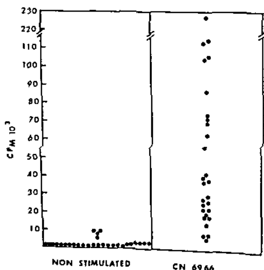


Fig 4 Methyl ³H TdR incorporation in human lymphocytes from 28 normal donors in response to CP (CN 6966 25 µg/ml) Each point is the mean of triplicate cultures in one experiment assayed on day 5 of lymphocyte culture

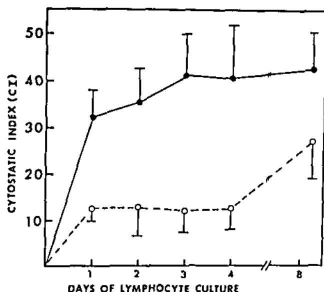
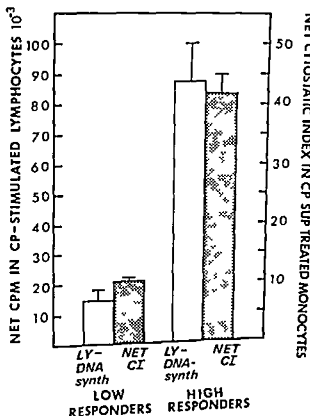


Fig 5 Kinetics of lymphokine release from human lymphocytes during the last 24 hours of the culture period indicated Lymphokine activity assayed on allogeneic monocytes cultured for 3 days before supernatant addition and expressed as cytostatic index of supernatant treated monocytes relative to monocytes cultured in HS M
O——O Non stimulated lymphocyte supernatants
●——● CP stimulated (CN 6966 25 µg/ml) lymphocyte supernatants
Mean ± S E M
n = 5



lymphokine activity was present after the first 24 hours of lymphocyte stimulation at a time when CP induced DNA synthesis was not detectable Supernatants from non stimulated lymphocytes also contained slight monocyte activating activity which increased with the time of lymphocyte culture

Correlation of lymphocyte DNA synthesis and lymphokine production in response to CP Lymphocyte donors were ranked in order of net cpm in CP stimulated lymphocytes and the bottom and top five were chosen as »low responders« and »high responders« in terms of lymphocyte DNA synthesis CP induced lymphokine production in the two groups is expressed by the difference in cytostatic index found in NS sup treated and CP sup treated monocytes calculated as net CI

(CI in CP sup treated monocytes – CI in NS-sup-treated monocytes)

This was done to correct for the slight spontaneous lymphocyte DNA synthesis and lymphokine production found in some experiments The results shown in Fig 6 indicate a relationship between lymphocyte DNA-synthesis and lymphokine production in response to CP

Effect of supernatants from autologous and allogeneic CP-stimulated lymphocytes on monocyte activation There was considerable variation in the lymphokine induced monocyte mediated cytostasis obtained in different experiments This might be due to differences in lymphokine activity of lymphocyte supernatants differences in monocyte responsiveness or low levels of reproducibility in the experimental system

To evaluate this lymphocytes from the same donor were tested for lymphokine production on different occasions and lymphocyte supernatants produced in one experiment were tested on different monocyte populations The results of 15 separate

Fig 6 Correlation of lymphocyte proliferation response and lymphokine release from CP stimulated lymphocytes
□ Methyl ^{3}H TdR incorporation in CP stimulated (CN 6966 25 µg/ml) lymphocytes cultured for 5 days expressed as (cpm in CP stimulated lymphocytes – cpm in non stimulated lymphocytes)
■ CP induced lymphokine activity in lymphokine supernatants expressed as net CI (CI in CP sup treated monocytes – CI in NS sup treated monocytes)
Monocytes cultured for 3 days before addition of supernatant
Mean ± S E M
n = 5 in each group

TABLE 6 *Act at on of Monocytes by Supernatants of Autologous or Allogene c CP st m lated Lymphocytes*

Lymphocyte donor	No of supernatants exam ned	Cytostatic index in separate experiments			
J H	4	56 7	57 6	45 9[a]	54 4[a]
T J	2	59 9	58 5	59 0[a]	
J B	l	38 3	31 1[a]		
E S	1	41 1[a]	37 2[a]		
S H	l	42 3	36 3		
N N	1	33 0[a]	27 2[a]		

ch figure is the mean of triplicate determinations in e experiment.

Allogeneic monocytes as effector cells

onocytes cultured for 3 days before addition of pernatant.

xperiments (Table 6) indicate that the amount of nonocyte activation induced expressed as cytostatic ndex is dependent on lymphokine activity in the supernatant A given supernatant seems to produce about the same level of activation in both autolo gous and allogeneic monocytes and the assay seems to be surprisingly reproducible

DISCUSSION

The activation of human mononuclear phagocytes to increased tumour cell cytotoxicity by mediators from stimulated human lymphocytes has to our knowledge been described only once previously by *Gougerot et al* (13) in a relatively long term (24 h) [51]Cr release microassay The reproducible (in each of 32 experiments) activation obtained in our system makes it possible to investigate further the mechanisms of lymphokine induced mononuclear phagocyte activation in a completely human *in vitro* system

The results are in general agreement with data from

... al origin (13) The mechanisms by which lymphokines activate macrophages and macro phages kill tumour cells are surprisingly poorly understood considering the extensive research in this field A wide array of secretory products released from macrophages has been described some of these interfering with thymidine incorpora tion assays (26) others actually lysing tumour cells (6 9)

Our failure to demonstrate cytostatic activity in the supernatants of activated monocytes is by no means conclusive since such factors may be labile or inhibited by serum components but stable factors such as cold thymidine can be excluded

The control experiments with intensive washing and trypsin treatment of activated monocytes indicates that the cytostatic influence demonstrated is not due to contaminating lymphocytes in the monolayer or to passive adsorption of lymphotox ins or antibodies to monocyte membranes Resis tance of lymphokine activation to trypsin has been described in a guinea pig system (7) and reversal of the activation of *in vivo* CP activated macrophages by 48 hours of *in vitro* culture reported in a mouse system (25) The effect of exposing target cells to monocytes disrupted by freezing and thawing is more difficult to interpret but the inhibition produced by disrupted supernatant-activated mono cytes suggests that lymphokine activation leads to accumulation of toxic molecules in the monocytes Their possible nature and origin remains to be determined

Lymphokines seem to be able to activate human monocytes at all stages of *in vitro* differentiation but a quantitative analysis of the lymphokine responsiveness of monocytes at different stages is difficult in this type of assay Differences in lymphokine responsiveness of macrophage popula tions have been described in animals (28) and experiments are in progress to examine this question in man

All 28 lymphocyte donors tested responded to CP by *in vitro* DNA-synthesis thus indicating a mitogenic effect of CP on human lymphocytes as demonstrated by *Godal et al* (12) The broad range of concentrations giving almost maximal response was also noted by *Godal et al* and indicates that CP may have unusual characteristics as a lymphocyte mitogen The lower end of the plateau is well within the range obtainable with the doses employed in clinical work with CP (23) even when not considering the antigen accumulation that probably occurs at reactive sites such as the liver and the spleen (29) The variation of the response may among other things be related to a secondary response being superimposed on the mitogenic response in sensitized individuals as many normal individuals have antibodies to CP (35) The lymphokine production in response to CP correlated with the magnitude of DNA-synthesis in the lymphocytes thus raising the possibility that sensitized individuals may respond to CP with higher levels of macrophage activation as has been demonstrated in animal systems (4) However all lymphocyte donors responded to CP with detectable

lymphokine release, thus indicating that the need for *in vivo* sensitization required in animals in order to obtain *in vitro* lymphokine release by CP-stimulation (5) does not apply to human systems The dissociation observed in the kinetics of lymphokine release and DNA synthesis in stimulated lymphocytes has been described previously for migratory inhibitory factor (MIF) (3) macrophage activating factor (MAF) (13) and lymphotoxin (19) the last two in human cells

In animal systems, CP has been reported to activate macrophages without lymphocyte cooperation (5) The results of experiments undertaken to examine whether this observation can be extended to human cells will be presented in a later publication

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ISOLATION OF ENZYMATICALLY DERIVED FRAGMENTS OF PORCINE IgG AND AN EXAMINATION OF THEIR REACTIVITY AGAINST STAPHYLOCOCCAL PROTEIN A

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Endresen C Isolation of enzymatically derived fragments of porcine IgG and an examination of their reactivity against staphylococcal protein A Acta path microbiol scand Sect. C 87 177–183 1979

Papain digestion of porcine IgG in the absence of cysteine resulted in a rather poor yield of fragments (less than 5 per cent) In the presence of cysteine 70 to 80 per cent of the IgG was degradated in 4 h Fragments with molecular weight of about 100 000 and 50 000 were separated by gel filtration The minor fraction (mol wt 100 000) most probably consisted of $F(c)_2$ fragments Fab/c fragments with both Fc and Fab determinants and also probably some $F(ab)_2$-like fragments The $F(c)_2$ fragments appeared to be a dimer of Fc stabilized by disulphide bonds The second main fraction (mol wt 50 000) contained Fc and Fab fragments Mild reduction of the Fc fragments resulted in Fc subfragments of different sizes thus indicating that papain cleavages had occurred on different spots in the Fc cha n Non reduced Fc fragments therefore seem to consist of several Fc subfragments stabilized by disulphide bonds The protein A reactivity of the isolated Fc fragments were rather low compared to the reactivity of intact IgG respectively 5–15 and 90 per cent. In addition protein A reactive Fab fragments were isolated from normal porcine IgG

Key words Porcine IgG staphylococcal protein A

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The proteolytic degradation of IgG into macromolecular fragments has been widely used in studies aimed at defining the location and structural basis of the various biological characteristics of antibody molecules

Earlier studies of isolated enzymatically derived Fc subfragments from human guinea pig and rabbits have shown that their binding to protein A were dependent on C_H2-C_H3 interaction No fragments so small that they contained only one domain showed any binding capacity to protein A (2 3 4 5) Normal porcine IgG as well as normal IgG from most mammalian species react with staphylococcal protein A through the Fc part of the γ-globulin molecule (6 9 12 13 16)

It has earlier also been described that traces of Fab fragments isolated from normal human and guinea pig IgG showed reactivity against protein A (8) and recently similar reactivity is found in normal Fab fragment isolated from rabbit IgG (2)

This report deals with the isolation and partial characterization of porcine IgG subfragments and examines their interaction with protein A

MATERIALS AND METHODS

Purification of porcine IgG from pooled normal sera was performed by ammonium sulphate precipitation followed by chromatography on a diethylaminoethyl cellulose (DE 32 Whatman England) column (3 6 × 52 cm) [illegible]

was concentrated by pressure dialysis to concentrations of 15–20 mg per ml and stored at −22°C if not used immediately

Papain digestion of IgG was carried out using 2 × crystallized papain (Sigma, USA) in 0 1 M phosphate buffer, 0 01 M EDTA-Na_2, pH 7 0, with a papain substrate ratio of 1 100 (w/w) Proteolysis was performed with and without cysteine (0 01 M) In some experiments the proteolysis was stopped by adding iodoacetamide or N-ethylmaleimide 50 per cent greater than the cysteine concentration or, when cysteine was omitted to 0 01 M concentration

Separation of Fab and Fc was performed using a column containing Whatman DE-32 cellulose equilibrated with 0 01 M phosphate buffer, pH 8 0 Samples were dialysed against the same buffer prior to chromatography Most of the Fab material was eluted by raising the ionic strength of the buffer to 0 045 M The Fc material was eluted by using a linear gradient ranging from 0 045 M to 0 3 M Na-phosphate buffer, pH 8 0

Gel filtration of papain digests was performed on columns of Sephadex G-150 (Pharmacia, Sweden) (3 2 × 91 cm) equilibrated with 0 1 M Tris-HCl, 0 2 M NaCl, 2 mM EDTA Na_2 and 0 02 per cent sodium azide, pH 7 6 The columns were operated upwards using a pumping speed of 15 ml/h Fractions (8 ml) were collected in a time-operated fraction collector The K_{av} (average participation coefficient) values of the fractionated proteins were calculated according to *Laurent & Killander* (15)

F(ab')$_2$ fragments were prepared by digestion of purified porcine IgG with pepsin (Sigma) in a 0 1 M acetate buffer, pH 4 0 containing 5 mM NaCl incubation 16 h at 37°C the substrate to enzyme ratio being 100 3 (w/w) The digest was then fractionated on a column of Sephadex G-150 equilibrated with 0 1 M Tris HCl buffer pH 7 8 containing 0 2 M NaCl and 2 mM EDTA-Na_2

Fab fragments were prepared by reduction of isolated F(ab')$_2$ fragments in 0 2 M 2 mercaptoethanol (pH 8 0–8 5) incubated at room temperature for 18 h followed by alkylation in 0 3 M iodoacetamide for 3 h at 37°C, and finally dialysis against saline buffered to pH 7 2

pFc fragments were prepared by digestion of purified IgG with pepsin (Sigma) in a 0 1 M acetate buffer pH 4 5, containing 5 mM NaCl incubation 18 h at 37°C the substrate to enzyme ratio being 100 1 (w/w) The digest was fractionated on a column of Sep[illegible]dex G-150 as described above and then the fraction containing the pFc' fragments was concentrated and further gel filtrated on a Sephadex G-100 column equilibrated with 0 1 ammonium carbonate buffer, pH 8 0 The main peak containing the pFc' fragments was tested in double diffusion analysis against rabbit anti porcine Fab' and rabbit anti-porcine Fc serum

Reduction and alkylation of the protein samples were performed as described in (19)

Apparent molecular weights of the IgG fragments and their polypeptide chains after reduction and alkylation were determined by the method of *Andrews* (1) using the K_{av} values estimated as described by *Laurent & Killander* (15)

Concentration of protein was carried out by ultrafiltration using Amicon cells and UM-2 filters (Diaflo the Netherlands), and the protein concentration determined as described in (17)

Antisera to porcine serum IgG, Fab' and Fc fragments were obtained from rabbits given 2 intramuscular injections of antigen in complete Freund s adjuvant (Difco, USA), 2 weeks apart Two booster injections (2 mg each) were given subcutaneously, 8 and 10 weeks after the first injection Blood was drawn from the marginal ear vein 6 to 7 days after each booster injection Commercial goat anti porcine serum and goat anti porcine IgG serum (Behringwerke AG, West Germany) were also used

Immunoelectrophoresis was performed using special Agar Noble (Difco) in barbitone buffer (I 0 025 pH 8 6) and a voltage of 6–8 V/cm

Double diffusion in agar was carried out as described by *Ouchterlony* (20) in 1 per cent Agar Noble (Difco) in barbitone buffer (0 025, pH 8 6)

Sodium dodecylsulphate (SDS) polyacrylamide gel electrophoresis (PAGE) was performed according to *Laemmli & Favre* (14) using 10 or 12 5 per cent gel

Sepharose-protein A was prepared as described in (21)

RESULTS

Only the IgG material which was eluted from the DEAE-cellulose column with 0 02 M Tris HCl buffer, pH 8 0 was examined in this study Analyses of the isolated IgG by immunoelectrophoresis revealed only one arc without any spur against both commercial goat anti porcine serum, goat anti porcine IgG serum, and antisera raised in rabbits against porcine serum and isolated IgG Approx 90 per cent of the isolated IgG was examined using a digestion time from 1 to 16 h The digestion was performed at pH 7 0 with and without cysteine The results of the gel filtration on Sephadex G-150 and the immunoelectrophoretic analysis of the digests are shown on Fig 1 and 2, respectively Only a minor fraction of the IgG was split by papain in the absence of cysteine (Fig 1 A, B) However, with cysteine present, approx 70 per cent of the IgG was

Fig 1 Gel filtration on Sephadex G 150 (3 2 × 93 cm V_0 241 ml) of the papain digest of porcine IgG without cysteine (A and B) and with cysteine (C and D) The column was equilibrated with 0 1 M Tris-HCl buffer pH 7 8 containing 0 2 M NaCl 2 mM EDTA Na_2 and 0 02 per cent sodium azide Elution rate 15 2 ml per h

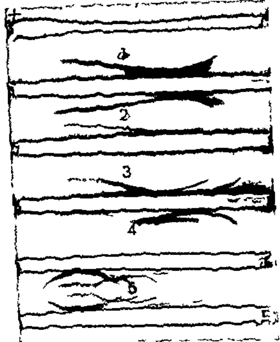


Fig 2 Immunoelectrophoresis of papain digest without cysteine (1 and 2) and with cysteine (3 and 4) Well 5 contains normal porcine serum Troughs 1 3 5 and 6 numbered from the top were filled with rabbit anti normal porcine serum troughs 2 and 4 were filled with rabbit anti porcine IgG

digested already after 4 h After 16 h some more IgG was split but as shown by immunoelectrophoresis (Fig 2) the Fc line was weaker and more diffuse indicating that also the Fc fragments had been further degradated In the 16 h digest an anodic fragment could be seen (Fig 2) This line most probably represents the Fc fragment which could not always be detected in the 4 h digest

Papain digestion in the presence of cysteine gave also a minor fraction (K_{av} 0 23) which has an elution volume corresponding to a mol wt of about 100 000 Both $F(ab')_2$ and Fc fragments reacted partially with this fraction against specific rabbit anti IgG serum (Fig 3) The K_{av} 0 23 fraction was applied to an immunosorbent column containing Sepharose with fixed specific rabbit anti porcine Fab antibodies About 40 per cent of the applied material reacted with the immunosorbent and after elution from the column this portion still reacted with both anti Fab and anti Fc sera in double diffusion analysis The non reactive portion, however reacted only with anti Fc serum Thus it seems that the material non reactive to anti Fab corresponds to dimers of Fc fragments The reactive

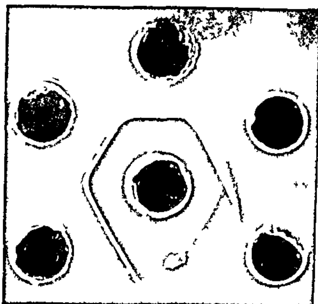


Fig 3 Immunodiffusion of the material with K_{av} = 0 23 (Fig 1)filled in well (1) porcine IgG (2) purified F(ab)$_2$ (3) purified Fc (4) rabbit anti porcine IgG serum (5)

fraction containing both Fab and Fc determinants may correspond to the Fab/c fragment isolated by papain digestion of normal human IgG as described by *Michaelsen & Natvig* (18) However the presence of some F(ab)$_2$ fragments cannot be excluded (18)

The peak eluted from the Sephadex G 150 with a K_{av} of 0 41 containing Fab and Fc fragments was concentrated and chromatographed on a DEAE-cellulose column The Fab fragments were eluted in one peak (A) whereas the Fc fragments were found

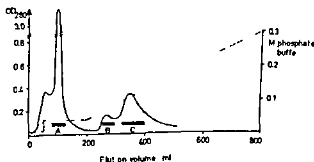
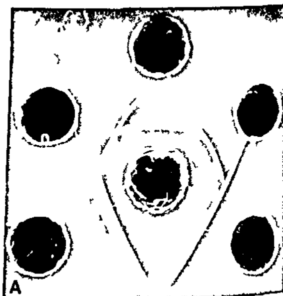


Fig 4 DEAE ion exchange chromatography of the Fab and Fc containing fraction (K_{av} 0 41) The column (1 5 × 30 cm) was equilibrated with 0 01 M sodium phosphate buffer pH 8 0 Fab fragments were eluted off the column with 0 045 M sodium phosphate buffer The Fc material was released using a linear gradient of 300 ml 0 045 M buffer in the mixing chamber and 300 ml 0 3 M sodium phosphate buffer pH 8 0 in the reservoir chamber Elution rate 20 ml per h The fractions were pooled as indicated

in 2 peaks (B and C) (Fig 4) Analysis of the Fc material isolated from peaks B and C by double diffusion and immunoelectrophoresis against diffe rent antisera did not indicate antigenic differences (Fig 5) Only minor differences in the peptide patterns were observed by SDS-electrophoresis of mercaptoethanol reduced samples (Fig 6) Determi nation of the mobilities in SDS-electrophoresis showed that the approx mol wt of the ma n



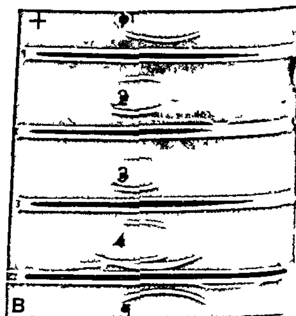


Fig 5 A Double diffusion analysis of material from ... IgG [illegible]

5) Fc (peak B) (2) Fc (peak C) (3) and the papain d gest of porcine IgG (4) Troughs 1 2 and 4 numbered from the top were filled with rabbit anti porcine IgG the third trough was filled with rabbit anti Fc serum

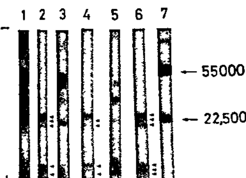


Fig 6 SDS polyacrylamide gel electrophores s of the Fc fragments non reduced and reduced with 5 per cent mercaptoethanol (1) Fc (peak B) (2) Fc (peak B) reduced (3) Fc (peak C) (4) Fc (peak C) reduced (5) Fc (peak B) prote n A reactive (6) Fc (peak B) protein A reactive and reduced and (7) reduced and alkylated IgG

peptides ranged from 25 000 to 10 000 SDS electrophoresis of non reduced newly prepared Fc fragments indicated the presence of some fragments with mol wt of around 45 000 to 50 000 34 000 24 000 to 18 000 and 10 000 However during storage at 4°C for a few days in concentrated solutions the Fc fragments apparently formed stable d mers which behaved like a 100 000 fragment by SDS-electrophoresis and by gel filtra tion on Sephadex G 200 in 5 M guanidine HCl 1 M HAc When the same material was concentrated reduced alkylated and gel filtered on a Sephadex G 200 in 5 M guanidine HCl 1 M HAc 3 peaks (I,II III) were formed (Fig 7)

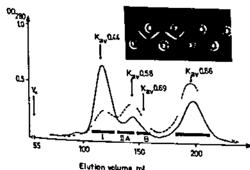


Fig 7 Gel Filtration on Sephadex G 200 of reduced Fc The column (2 5 × 43 cm V_o 55 2 ml) was equ l brated and eluted with 1 M HAc 5 M Guanid ne HCl Elution rate 2 8 ml per h The fractions were all bulked as ind cated The Fgure also shows double d ffus on analysis of material from the peaks well (1) conta ns fraction I (2) fraction II A (3) fraction II B (4) fraction III (6) pFc fragments (5 7 and 8) rabbit anti porcine IgG

TABLE I *Protein A Reactivity of Porcine IgG and Various Subfragments of Porcine IgG as Est mated on a Sepharose Protein A Column*

Fractions	Protein A reactivity per cent
IgG	90–92
Fab (peak A Fig 4)	2–5
Fc (peak B Fig 4)	13–15
Fc (peak C Fig 4)	5–7
Fc subfragment (peak I Fig 7)	0
Fc subfragment (peak II A Fig 7)	0
Fc subfragment (peak II B Fig 7)	0

Peak I seemed to contain fragments ranging in size from mol wt 24 000 to 20 000 and peak II fragments in the range of mol wt 18 000 to 10 000 Peak III contained material which accor ding to the K_{av} (0 86) should have mol wt of approx 6 000 This material gave no precipitation with the antisera (Fig 7) and was not further examined Peak II was divided into 2 fractions called II A and II B Double diffusion in agar showed that peak I and fraction II A shared antigenic determinants Fraction II B seemed mostly to consist of material of the C_H3 domain but contaminated fragments from the C_H2 cannot be excluded

All fragments isolated were tested for protein A reactivity as previously described (5) Samples (2–3 mg protein) were passed through a Sepharose

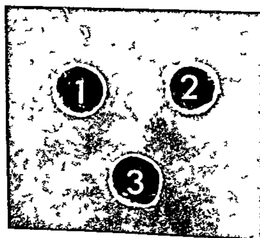


Fig 8 The »star« formation between prote n A (1) normal rabb t IgG (2) and protein A reactive normal porcine $F(ab')_2$ (3)

protein A column with a binding capacity of 4 8 mg purified human Fc The results are shown in Table 1 There was not only an interaction of whole IgG and parts of the Fc material with protein A, but also some of the papain derived monovalent Fab (from peak A, Fig 4) and some of the pepsin-derived F(ab')$_2$ fragments fixed to the Sepharose protein A column Furthermore, concentrated F(ab')$_2$ fragments (5 mg/ml) produced a »star« together with protein A and normal rabbit IgG (Fig 8) Monovalent Fab or Fab' fragments showed no reaction, the reaction thus being dependent on the divalent F(ab')$_2$ fragments

DISCUSSION

The normal porcine IgG isolated in this study has not been examined for subclasses By using similar isolation methods, *Kaltreider & Johnson* (11) found that the IgG from immunized animals which was eluted off the DEAE-cellulose column with 0 02 M Tris-HCl buffer, pH 8 0, consisted of IgG 1 and IgG 2 The other subclasses present, IgG 3 and IgG 4, were retarded on the column and could be eluted by increasing the ionic strength of the buffer Most probably the isolated IgG preparation examined in this study consists of the subclasses IgG 1 and IgG 2 These 2 subclasses cannot be separated by immunoelectrophoresis (11)

As shown the proteolysis of porcine IgG with papain is dependent on the presence of cysteine, and can thus be compared to that of human IgG 2 and IgG 4 (7, 10) An explanation for this can be that the folding of the polypeptide chains in the IgG masks the site(s) of proteolysis with papain The cleavage of some disulphide bridges in the heavy chains by a reducing agent such as cysteine leads to exposure of sites in the hinge or near to the hinge region which are susceptible to the enzyme In addition to cleavage near the hinge region it is also obvious that papain splitting also occurs at sites along the Fc chain as shown by SDS electrophoresis of non reduced and reduced Fc preparations Most probably, smaller peptide units of Fc are stabilized by intra domain disulphide bridges

Gel filtration on Sephadex G 150 columns and electrophoresis of non reduced Fc showed the presence of fragments with mol wt in the range of 45,000 to 50 000 and some dimer Fc with mol wt near 100 000

The dimer formation of the Fc fragment must be due to intra molecular disulphide bond interchanges since – especially in solution with high concentration of Fc – they moved as a single peak by gel filtration under denaturating conditions with a K_{av} value corresponding to a mol wt of 100,000

Approx 90 per cent of the isolated IgG reacted with protein A, the reactivity of porcine IgG thus being comparable to that of human and guinea pig IgG (4, 5) However, in contrast to the Fc fragments isolated from human and guinea pig IgG the purified Fc fragments from porcine IgG showed rather low reactivity (approx 5–15 per cent) This may be due to structural alterations of the Fc chains caused by papain splitting

Very slight differences in the peptide patterns of protein A non-reactive and protein A reactive Fc preparations were observed by SDS-electrophoresis (Fig 6) However, it is possible that the non reactive fragments have lost short peptides which are of importance for the protein A reactivity This reactivity has previously (5) been found to be dependent on a complete Fc chain No direct precipitation was observed between isolated fragments and protein A The Fab/c like fragments isolated, containing both Fab and Fc structures, gave no precipitation in agar with protein A at a concentration of about 10 mg per ml

As described earlier, traces of F(ab')$_2$ fragments isolated from normal human guinea pig and rabbit IgG (2, 8) were protein A reactive This was also true for a small portion of normal porcine F(ab')$_2$ fragments as well as papain-derived Fab fragments The F(ab')$_2$ fragments formed a »star« together with protein A and normal rabbit IgG (Fig 8) as described earlier using human and guinea pig normal F(ab')$_2$ If this reactivity is due to the presence of traces of IgG in normal sera with immunogenic specificities against protein A or is due to a small portion of IgG present containing a protein A non immune reactive determinant in the Fab region, remains to be demonstrated

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THE BINDING TO PROTEIN A OF IMMUNOGLOBULIN G AND OF Fab AND Fc FRAGMENTS

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Endresen C The binding to protein A of immunoglobulin G and of Fab and Fc fragments Acta path microbiol scand Sect. C 87 185–189 1979

Results are presented to show that protein A (pA) may fix to the Fab region of IgG outside the antigen binding site Thus pA reactive Fab fragments were isolated both from specific anti-egg albumin and specific anti measles virus antibodies A probable significance of this Fab reactivity in the precipitation reaction between IgG and pA is discussed Reactive IgG from human guinea pig rabbit, and porcine normal sera all showed similar avidity towards pA Slight differences between IgG subclasses were however observed The avidity of pA reactive Fab and Fc fragments isolated from normal porcine IgG was highest in the latter

Key words Immunoglobulin G Fc and Fab fragments protein A binding

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The interaction between protein A (pA) from *Staphylococcus aureus* and IgG from a number of mammalian species is well documented (9 10 13 14 17) This interaction is explained as a binding of pA to the Fc re...

...ragments containing an intact Fc chain were pA reactive (4) Similar results were obtained by the examination of Fc from IgG of guinea pig (3) rabbit (1) and porcine sera (2) In addition to the pA Fc interaction *Grov & Endresen* (8) showed that pA reacted with traces of normal F(ab')$_2$ fragments isolated from human and guinea pig IgG These normal pA reactive F(ab')$_2$ fragments could replace immune F(ab')$_2$ isolated from rabbit anti pA sera in the »star« reaction (11) Traces of pA reactive Fab fragments have also been obtained from normal rabbit IgG (1) and porcine IgG (2)

The present study deals both with the avidity of the interaction between pA and normal IgG from human guinea pig porcine and rabbit sera and isolated Fab and Fc fragments from porcine IgG as well as with the nature of the pA reactive Fab fragments

MATERIALS AND METHODS

Normal human rabbit guinea pig and porcine IgG were isolated from pooled sera as previously described (1 2 3 4)

Guinea pig anti egg albumin (EA) antiserum was produced as described in (7)

Guinea pig anti measles virus antiserum was prepared by ...

... were bled one week after the last injection

All other antisera used were obtained from Behringwerke (BRD) or were made in our laboratory as described earlier (1, 2, 3, 4)

Guinea pig IgG$_1$ and IgG$_2$ were isolated as described in (15) and its specific anti EA and anti measles virus antibodies were isolated on a column of Sepharose EA (7) and absorption with measles virus (20) respectively

F(ab')$_2$ and Fab fragments were prepared as described in (16, 19)

Porcine Fab F(ab')$_2$ and Fc were obtained as described in (2)

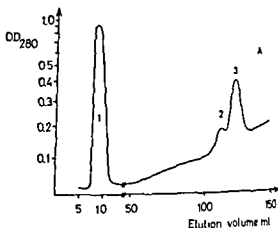
Sepharose 4B-protein A (Seph-pA) was made as previously described (7) Sepharose 4B CL-protein A obtained from Pharmacia (Uppsala Sweden) was also used The columns (1 × 10 cm) were equilibrated with PBS After application of the sample the column was washed with 50 ml PBS and then connected to the gradient system which consisted of 50 ml PBS in the mixing chamber and 50 ml 3 5 M sodium thiocyanate (NaSCN) in the reservoir chamber The fractions were collected in 1 ml portions

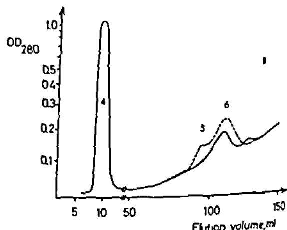
Diffusion in agar and immunoelectrophoresis were performed as described earlier (4)

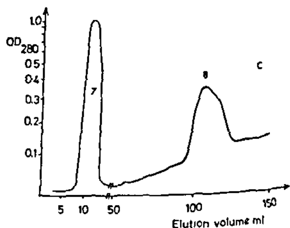
RESULTS

Normal sera from man, rabbit, guinea pig and swine, and a guinea-pig-immune serum, were analysed for both pA binding and conditions for elution, using a gradient system of 0–3 5 M sodium thiocyanate The elution diagrams are presented in Fig 1 The immunoelectrophoretic analysis of bound and unbound material is presented in Fig 2

Absorbed IgG material from all sera was eluted using NaSCN concentrations of between 1 4 and 2 7 M The elution profiles especially of IgG from normal human and immune guinea pig sera, indicated some heterogeneity in the affinity of IgG for pA (Fig 1, A and B) The IgG material in normal human serum and the IgG in the guinea pig immune serum which contains more of the IgG$_1$ subclass than normal guinea pig serum, eluted in two partially separated peaks The immunoelectro-







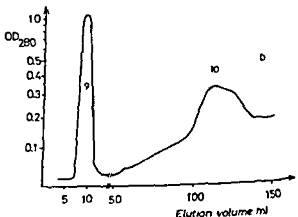


Fig 1 Chromatography of normal sera from human (A), guinea pig (B) rabbit (C), and porcine (D) origin on a Sepharose protein A column (1 0 × 10 cm) The sample volumes were 1 ml, with the exception of rabbit serum, which was 2 ml In addition to normal sera a guinea-pig anti-egg albumin (EA) was also analyzed (B dotted curve) Bound protein was eluted off using a gradient of increasing molarity of NaSCN (0–3 5)

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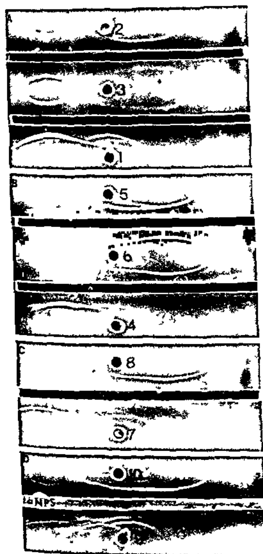


Fig 2 Immunoelectrophoresis of reactive and non reactive material of human (A) guinea pig immune serum (B) rabbit serum (C) and porcine serum (D) The different fractions analyzed numbered from 1 to 10 are indicated in Fig 1 The antisera used are rabbit anti normal human serum (a NHS) rabbit anti normal guinea pig serum (a NGS) goat-anti normal rabbit serum (a NRS) and rabbit anti normal porcine serum (a NPS)

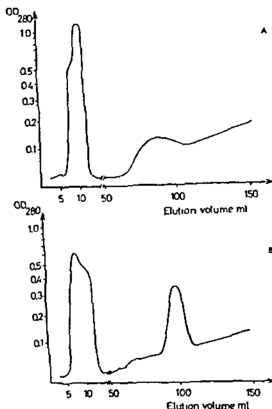


Fig 3 Chromatography of Fab (A) and Fc (B) isolated from normal porcine IgG on a Sepharose protein A column

concentrations of NaSCN as shown in Fig 3 The Fab fragments however showed some heterogeneity with regard to pA binding since they eluted over a rather wide range of NaSCN concentrations Rechromatography of the bound proteins demonstrated similar patterns of binding No difference was observed when the Fab and Fc fragments were applied to the column in a mixture or when they were tested separately Isolation of guinea pig IgG_1 and IgG_2 was performed by a combination of ammonium sulphate precipitation and DEAE-cellulose chromatography (15) IgG_1 antibodies with specificity against EA were isolated using a Sepharose column with covalently bound EA The isolated IgG_2 and IgG_1 the latter having an immune specificity against EA both precipitated pA in agar, as did purified IgG_1 having a specificity against measles virus Pepsin digestion of the purified

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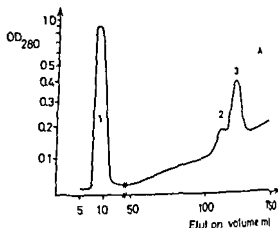
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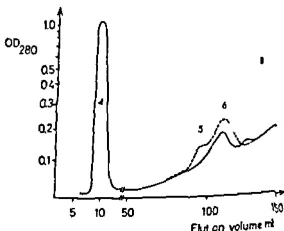
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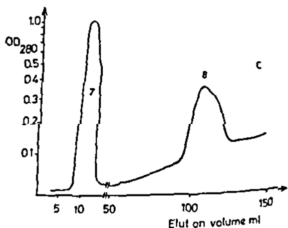
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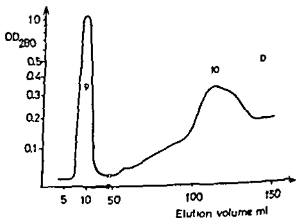


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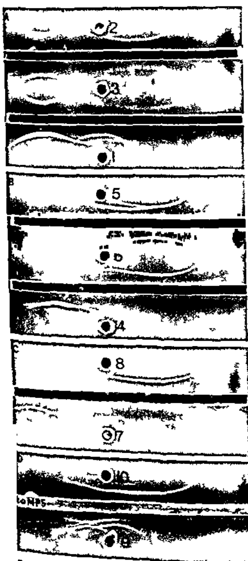


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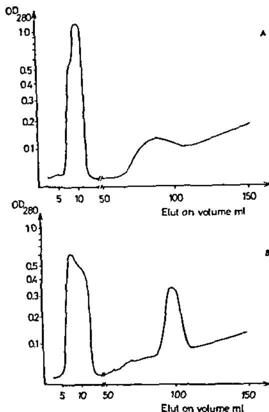


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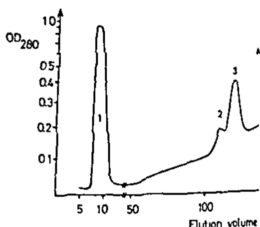
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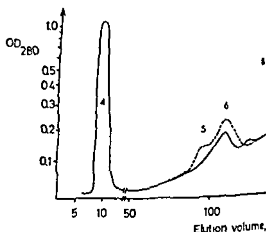
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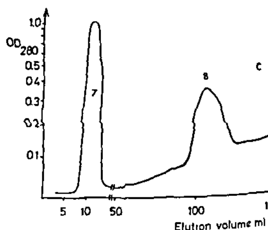
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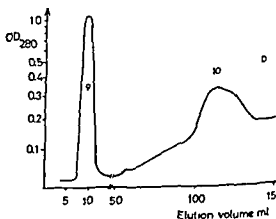


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The $F(ab'\gamma_1)_2$ fragments with specificity against EA or measles virus gave, after concentration to 20–25 mg/ml, a »star« together with pA and normal rabbit IgG as earlier shown for guinea pig $F(ab'\gamma_2)_2$ fragments isolated from normal guinea pig sera (8) A mixture of $F(ab'\gamma_1)_2$ and normal rabbit IgG coprecipitated with pA in agar

DISCUSSION

A range of salt concentrations between 1 4 and 2 7 M was used for the elution of immunoglobulins from all species tested The results indicate a similarity in nature and in the binding forces between pA and IgG from all these species The results of testing the binding of immunoglobulins to Seph-pA showed some differences in the avidity of IgG subclasses The difference between IgG_1 and IgG_2 from guinea pig was the most pronounced, but also subclasses of human IgG showed a slightly different avidity against pA (Figs 1 A and B, 2 A and B)

The dominant reaction between pA and normal IgG is the Fc interaction It was shown that it was necessary to use an almost identical salt concentration to split the binding between pA and porcine Fc as was needed for intact IgG The nature of the Fab interaction with pA seems to be weaker and somewhat heterogeneous since the Fab fragments eluted over a rather broad range of salt concentrations (Fig 3 A)

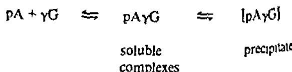
Recently, *Mackenzie et al* (18) described that only Fc and not $F(ab')_2$ fragments isolated from murine IgG, fixed to the Seph pA column However, it has been shown by *Kronvall et al* (12) working with similar murine myeloma IgG, that only one of these murine myeloma IgG precipitated pA, the others either did not react at all or else showed only inhibition reactivity The amount of IgG having Fab reactivity seems to correlate with the precipitating capacity of the serum The amount of IgG having Fab reactivity found in normal rabbit serum was smaller than that of normal porcine serum (1, 2) Normal porcine sera precipitate pA very well, normal rabbit sera do not

The reason why *Mackenzie et al* (18) did not detect traces of Fab reactivity can be that they have used Fab from a pA non precipitating IgG

Normal guinea pig IgG, consisting mostly of subclass IgG_2 as well as IgG_1 isolated from a guinea pig anti EA serum, is known to precipitate pA (5) Purified IgG_1 antibodies, specific against EA and measles virus also precipitate pA, and it could be shown that small portions of the $F(ab'\gamma_1)_2$ and $Fab\gamma_1$ fragments isolated from these specific antibodies reacted with pA These results strongly suggest that pA interacts with a region on the Fab fragments different from the true antigen binding site

To verify if the antigen binding site could be excluded, raising of anti-idiotypic antibodies in both guinea pigs and rabbits was attempted However after absorption of the antisera with pA non reactive $F(ab')_2$, no anti idiotypic antibodies were seen (personal observation) The Fab reactive site cannot be within the hinge region since both pepsin-derived $F(ab')_2$ and papain-derived Fab fragments are pA-reactive (2) It is known that the Fab fragments have lost most of the hinge region

A very important unsolved problem is the meaning of the Fab interaction in the precipitation reaction between pA and IgG It has earlier been shown that only the pA-reactive $F(ab')_2$ from normal IgG can replace immune $F(ab')_2$ in the »star« phenomenon (8) The main fraction of F(ab') fragments isolated (>95 per cent) was pA non reactive and gave no »star« (8) Furthermore only bivalent pA-reactive $F(ab')_2$ gave both »star« and coprecipitation (8) Since the pA-reactive F(ab') fragments isolated from normal IgG can substitute reactive $F(ab')_2$ from anti-pA, it is possible that normal IgG having a Fab reactivity like that of specific IgG antibodies is responsible for the precipitation reaction between pA and normal sera The Fab reactivity may be responsible for the secondary reaction suggested by *Kronvall et al* (13) who postulated a two step reaction for the precipitation of pA by human γ-globulin


$$pA + \gamma G \rightleftharpoons pA\gamma G \rightleftharpoons [pA\gamma G]$$

They were also able to show that the second reaction was different from the primary reaction which gave only soluble complexes (13)

The pA-binding site on Fab of normal IgG is probably of the same or similar nature as that of Fc fragments even though fragments from porcine IgG showed lower pA avidity of the Fab than of Fc

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Acta path. microbiol scand. Sect. C, 87 191–196 1979

THE HUMAN MIXED LYMPHOCYTE REACTION RESPONDER AND STIMULATOR CAPACITIES OF HIGHLY PURIFIED T- AND NON-T CELLS AND THE ROLE OF MONOCYTES

PETER HOKLAND and IVER HERON

Institute of medical microbiology Bartholin Building University of Aarhus DK 8000 Aarhus Denmark

Hokland P & Heron I The human mixed lymphocyte reaction Responder and stimulator capacities of highly purified T and non T cells and the role of monocytes Acta path microbiol scand Sect. C, 87 191–196 1979

E rosette sedimentation with AET treated sheep red blood cells and human lymphocytes provided a source of highly purified T and non T cells Together with unseparated cells these were tested for responder and stimulator capacities in the mixed lymphocyte reaction and it was found that the non T cells were weak but consistent responders only when T cells were present as stimulators On the other hand T T combinations always exhibited weak proliferation demonstrating that T cells possess stimulator capacities Adherent cells grown on Petri dishes were found to exert a dual function as helper cells in low and suppressor cells in high concentrations

Key words Human mixed lymphocyte reaction T and non T subpopulations Petri dish adherent cells

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The mixed lymphocyte reaction (MLR) has been used both as a test of histocompatibility and as an in vitro model of homograft rejection It has been established that the cells responding by proliferation in this assay are mainly T-cells (*Andersson et al* 1973 *Han et al* 1976 *Potter & Moore* 1977) and that most of the stimulatory capacity is found in the non T cell fr[illegible]

[illegible]

express surface immunoglobulin (*Philips & Weissrose* 1974) Furthermore, it has been amply demonstrated that T cells possess certain stimulatory capacities (*Lonai & McDevitt* 1977)

Another cell population of importance in the MLR is that of the monocytes *Alter & Bach* (1970) and *Blomgren* (1977) found that monocytes of stimulator or responder origin could restore the response of an MLR depleted of these cells Furthermore it has been shown that in the human monocytes themselves can exert potent stimulation (*Rode & Gordon* 1974)

Abbreviations
AET = S-(2 aminoethyl iso-thiouronium bromide) hydrobromide 99%
FCS = foetal calf serum
[illegible]
streptomycin (100 µg/ml)
MLR = mixed lymphocyte reaction
PBL = peripheral blood lymphocytes
PBS = phosphate buffered saline pH = 7 2
RPMI 1640 = Roswell Park Memorial Institute medium number 1640
SRBC = sheep red blood cells

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E rosette sedimentation with AET treated sheep red blood cells and human lymphocytes provided a source of highly purified T and non T cells Together with unseparated cells these were tested for responder and stimulator capacities in the mixed lymphocyte reaction and it was found that the non T cells were weak but consistent responders only when T cells were present as stimulators On the other hand T T combinations always exhibited weak proliferation demonstrating that T cells possess stimulator capacities Adherent cells grown on Petri dishes were found to exert a dual function as helper cells in low and suppressor cells in high concentrations

Key words Human mixed lymphocyte reaction T and non T subpopulations Petri dish adherent cells

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The *mixed lymphocyte* reaction (MLR) has been used both as a test of histocompatibility and as an in vitro model of *homograft* rejection It has been established that the cells responding by proliferation in this assay are mainly T-cells (*Andersson et al* 1973 *Han et al* 1976 *Potter & Moore* 1977) and that most of the stimulatory capacity is found in the non T cell f[illegible]

[illegible] mixed lymphocyte reactions (*Harrison & Paul* 1973 *von Bohmer et al* 1973) and that a proportion of the lymphoblasts in the human MLR express surface immunoglobulin (*Philips & Weisrose* 1974) Furthermore it has been amply demonstrated that T cells possess certain stimulatory capacities (*Lonai & McDevitt* 1977)

Another cell population of importance in the MLR is that of the monocytes *Alter & Bach* (1970) and *Blomgren* (1977) found that monocytes of stimulator or responder origin could restore the response of an MLR depleted of these cells Furthermore it has been shown that in the human monocytes themselves can exert potent stimulation (*Rode & Gordon* 1974)

Abbreviations
AET S-(2 aminoethyl iso thiouronium bromide) hydrobromide 99 %
FCS = foetal calf serum
[illegible]
[illegible] Institute medium number 1640
SRBC = sheep red blood cells

The aim of the present study was to further elucidate the role of pure T and non T cell fractions in the human MLR and to examine the role of monocytes in this reaction

MATERIALS AND METHODS

1 *Lymphocyte Isolation*

Lymphocytes from normal human blood donors were prepared by I F flotation (*Bøyum* 1968) of freshly drawn heparinized venous blood or buffy coat cells obtained following slow centrifugation of decalcified blood

The lymphocyte rich fraction was washed three times in medium 199 and centrifuged at 200 G for 8 min whereupon the cell concentration was adjusted to 1×10^7/ml Mean and standard deviation of lymphocyte recoveries during these isolations were 76 1 ± 3 2% (calculated from total leucocyte and Leishman-differential cell counts) The viability was always greater than 95% as judged by trypan blue dye exclusion

2 *Monocyte Identification*

Monocytes in cell suspensions were identified by their ability to phagocytose latex or fluolite particles (*Arvilommi* 1975) In rosette suspensions morphological criteria determined after acridine orange staining were used (*Hertel Wulff & Rubin* 1976) In the aforementioned isolations the mean percentage of monocytes was 14 0 (range 12–22)

3 *Removal of Phagocytic and Adherent Cells*

Treatment of I F cell suspensions with carbonyl iron powder and a magnet provided a source of monocyte depleted lymphocytes To each millilitre of cell suspension (1×10^7 per ml) 2 mg of carbonyl iron was added The mixture was carefully suspended in a 1 litre Roux bottle and incubated for 1 hour at 37°C in a 5% CO_2 environment The iron was collected with a magnet in one end of the bottle and the lymphocytes decanted off Decanting under magnetism was repeated twice The resulting cell suspension usually contained less than 0 3% monocytes and the lymphocyte yield was about 60% No significant changes in the relative proportions of lymphocytes with different markers were observed after this procedure

4 *Preparation of Monocytes*

Adherent cell monolayers were prepared on plastic Petri dishes (Nunc Roskilde Denmark) as described by *Koller et al* (1973) To each dish (d 55 mm) was added around 2×10^7 unseparated peripheral blood lymphocytes (PBL) in 7 ml of culture medium The adherent cell monolayers formed were rinsed with PBS after 24 48 and 72 h and the culture medium renewed after each rinse After the last renewal the adherent cells were dislodged by a rubber policeman and the cell concentration adjusted to 1×10^6/ml in culture medium The yield of monocytes after this procedure (calculated form the total number of monocytes present originally) ranged from 8 to 37% The purity ranged from 65–87% (mean 69%) the rest of the cells being lymphocytelike

5 *E rosettes*

SRBC (obtained weekly form Statens Seruminstitute Copenhagen Denmark) were washed four times in cold saline immediately before use and treated with AET (Sigma St Louis USA) as previously described (*Hokland et al* 1976)

Rosetting was performed by mixing equal volumes of AET SRBC (1% suspension) and lymphocytes (1×10^6/ml) After incubation at 37°C for 15 min and centrifugation at 250 G for 8 min the mixture was incubated at 4°C for at least 45 min After addition of acridine orange stain the percentage of rosette forming cells was determined by counting at least 200 viable cells under a Leitz Orthoplan phase contrast microscope equipped with an Osram HBO 200 mercury lamp and an Opak fluorvertical illuminator (E Leitz Wetzlar Germany) Acridine orange stain permits a distinction to be made between aggregated red cells and rosettes and between monocytes and lymphocytes (*Hertel Wulff & Rubin* 1976)

6 *Separation of T and non T Lymphocytes*

T lymphocytes were separated from non T cells by AET SRBC rosette sedimentation essentially as previously described (*Hokland et al* 1976) The purification is outlined in Fig 1 Briefly E rosettes were produced by incubating 3×10^6 lymphocytes in 2 5 ml medium 199 with 2 5 ml of a 1% AET SRBC suspension in medium 199 and treating them as described under (5) Before resuspension 1 5 ml of the supernatant was replaced by foetal calf serum (FCS) By means of a wide bore Pasteur pipette the rosettes were then layered onto 3 ml I F in a 10 ml test tube and centrifuged at approximately 650 G for 30 min This procedure separated T cells (pellet) from non T cells (interface) The rosetting procedure was repeated with the interphase cells after they had been pipetted off and washed twice in culture medium

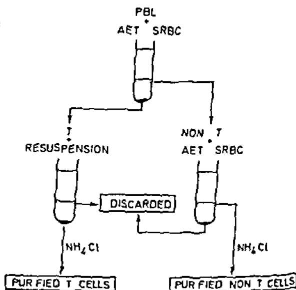


Fig 1 Purification of the T and non T lymphocyte fractions For further description see paragraph 6 in Materials and Methods

The T cells in the pellet were resuspended to 4 ml and stirred on a vortex mixer for 1–2 min Thereafter the E rosetting procedure was repeated In order to lyse red cells all cell suspensions were treated with 0 01 M Tris 0 83% NH_4Cl pH 7 2 at 37°C for 5 min After one wash in PBS and one in culture medium the cell suspens ons were ready for culturing The interfase of the second T rosette sedimentation was not used in the experiments subsequently described

7 *Culturing Conditions*

To 100 ml RPMI 1640 without L glutamine (Gibco)15 ml pooled heat inactivated male A serum (provided by the Blood Bank Municipal Hospital Aarhus) 1 ml Hepes buffer solution (Microbiological Associates) 1 ml non-essential aminoacids (Gibco) 10^5 IE penicillin and 100 µg streptomycin solution and 1 ml L glutamine (G bco) were added

MLR were performed in flat bottomed microtitre pla es (F

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... or 10^5 responder cells and 10^5 autologous irradiated stimulator cells (exp 5–7 Table 2) 14C

... and harvested onto glassfibre filters using a 12 channel semi automatic sample harvester (Skatron Lierbyen Norway) The filter strips were dried and added to 2 ml scintillation fluid in counting vials. Counting was performed in a Packard 2450 liquid scintillation spectrophotometer MLR and control cultures were done in triplicate cultures The median of these tripl cate cultures has been used if not otherwise stated

RESULTS

The T cell and non T cell fractions recovered from the separation procedure outlined in Fig 1 gave suspensions with viabilities over 95% and yields (expressed as percent of the maximum obtainable) ranging from 60–80%

In Table 1 the T cell markers (AET rosettes) of the fractions are shown after the first and second rosette sedimentation It can be seen that a relatively pure top fraction is obtained already after the first sedimentation (1 8% T-cell contamination) and that there is a further degree of purification in the bottom fr...

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TABLE 1 *Percentage of AET SRBC Rosettes in Lymphocytes Subpopulations before and after I F Flotations Mean and Standard Deviation is Shown*

Unseparated cells	78 4 ± 6 42
T-cells after one I F sedimentation	93 6 ± 5 19
T-cell after two I F sedimentation	98 7 ± 1 75
Non T cells after one I F sedimentation	1 8 ± 0 62
Non T cells after two sedimentations	0 4 ± 0 21

three experiments In two of them neither the responder nor the stimulator populations had been depleted for monocytes and the monocytes added were allogeneic In the third experiment, both

third experiment the optimum monocyte concentration is 10% whereas the optimum value in the two other experiments ranges from 12 to 40% In every case however a clear inhibition of the MLR response was seen when more than 50% monocytes were added

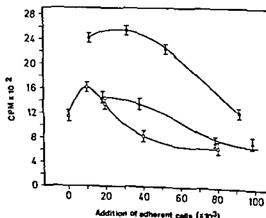


Fig 2 Thymidine incorporation in MLR cultures with graded addition of adherent cells recovered from Petri dish monolayers The number of monocytes before addition of Petri dish cells were □ 0 3 × 10^3 ● 10 2 × 10^3 and ▲ 18 1 × 10^3 Mean and standard deviation of triplicate cultures are shown Control cultures □ 377 cpm ● 763 cpm ▲ 42 cpm

TABLE 2 *DNA Synthesis in MLR's Combining Different Lymphocyte Subpopulations Numbers in Parenthesis are Stimulation Indices*

MLR combinations		Exp No						
Responder	Stimulator	1	2	3	4	5	6	7
Unsep	Control	135	255	45	266	81	253	44
	Unsep	1544 (11 5)	1197 (4 7)	1536 (34 1)	1552 (5 8)	3590 (44 3)	2537 (10 0)	6514 (148
	non-T	1355 (10 0)	1842 (7 2)	3086 (68 6)	NT	4100 (50 6)	7697 (30 4)	5458 (124
	T	834 (6 2)	535 (2 2)	1228 (27 2)	632 (2 4)	1060 (13 1)	1305 (5 2)	530 (12 0)
non T	Control	420	201	53	218	348	101	121
	Unsep	875 (2 1)	744 (3 7)	NT	464 (2 1)	1388 (4 0)	1544 (15 3)	774 (6 4)
	nonT	535 (1 3)	351 (1 8)	56 (1 0)	212 (1 0)	450 (1 3)	266 (2 6)	121 (1 0)
	T	921 (2 2)	821 (4 1)	283 (5 3)	272 (1 3)	919 (2 6)	2001 (19 8)	515 (4 3)
T	Control	314	134	54	189	199	487	45
	Unsep	1487 (4 7)	1005 (7 5)	1171 (21 7)	3064 (16 2)	1271 (6 4)	2396 (4 9)	4752 (105
	non-T	2105 (6 7)	1987 (14 8)	4181 (77 4)	2654 (14 0)	1418 (7 1)	6909 (14 2)	4789 (106
	T	619 (2 1)	398 (3 0)	1268 (23 5)	941 (5 0)	6167 (31 0)	2090 (4 3)	541 (12 0)

The responder and stimulator capacities of the purified lymphocyte fractions were tested in parallel with unseparated adherent cell depleted cells from the same donor, these had been subjected to the same treatment without addition of SRBC (gradient centrifugation – and NH_4Cl) as the purified subpopulations In each MLR combination 2×10^4 adherent cells from the responder cell donor was added to the culture These did not significantly influence the thymidine incorporation in the control cultures The data from seven such experiments are summarized in Table 2 It will be seen that unseparated cells were stimulated by all three stimulator cell preparations the non-T cell fraction giving the highest (Mean S I 48 5) and the T cell fraction the lowest counts (Mean S I 9 8), (significant stimulation defined here as stimulation indices greater than 2 0) In contrast to this the non-T cell fraction could be stimulated by the unseparated cells (Mean S I 5 6) and the T-fraction (Mean S I 5 7), whereas the non-T cell fraction did not show any stimulatory capacity (Mean S I 1 4) When an allogeneic non-T cell fraction was used as responder cells then similar results to those using unseparated cells were observed Again the T cell fraction showed stimulatory capacities (Mean S I 11 6) In experiments 5–7 comparisons were made between the different control procedures and it was found that addition of the autologous irradiated cells did not significantly increase the control values

DISCUSSION

The many contradictory reports on the identity of the cells responsible for stimulator and responder functions in the human MLR prompted us to investigate this problem further and the present study was designed to elucidate the role of T and non-T cell fractions in the MLR The method chosen to purify these subpopulations was the AET-SRBC method since we have already demonstrated that it is an efficient separation tool (*Hokland et al* 1976) The results of surface marker assays on the purified cells (Table 1) further support this in that the T cell fraction contained 98% AET-SRBC RFC and non-T fraction 0 4%

In an earlier report we demonstrated that a subpopulation of the non T cell fraction which did not form E rosettes with sheep erythrocytes sensitized with IgG antibody (EA-RFC) but possessed Ig on their surface were the most potent stimulator cells, whilst the T cell fraction were found to be the most potent responder cells (*Hokland et al* 1978) Because of the complexity of the interfase fraction we have deliberately used the expression non-T cell fraction thereby stressing that it contains several subpopulations of lymphocytes which to varying extents bear surface immunoglobulin, receptors for the Fc portion of IgG, and receptors for complement fractions

Our data (Table 2) demonstrate that the most efficient responders are the T cells and that the most efficient stimulators are found in the non-T cell

fraction, thus supporting several other reports (*Han et al* 1976 *Potter & Moore* 1977 *van Oers & Zeijlemaker* 1977 *Lohrmann et al* 1974a *Lohrmann et al* 1974b) Furthermore two interesting points emerge

First a consistent pattern of proliferation in the non T cell fraction is obtained Significant thymidine incorporation is seen only when T cells are present in the stimulator population i e in the combinations »B« + unseparated and »B« + T cells whereas »B« + »B« cells results in no detec

has been demonstrated in mice by *Harrison & Paul* (1973) and *von Bohmer* (1974) but it has not previously been observed in a human MLR DNA synthesis as a consequence of the recognition of responder cells by stimulator cells may occur as follows (1) the stimulator cell may itself synthesize DNA This is highly unlikely in our system because the stimulator cells were irradiated with 4500 rads (2) the cell may secrete a soluble blastogenic factor as a consequence of its recognition of the responder B cell this factor provokes B cell proliferation

Secondly our data indicates that T cells possess stimulatory capacities This can be seen from the fact that the combination T + T cells in all of the seven experiments exhibited significant stimulation (Mean S I 11 6) These results are not in agreement with *Han et al* (1976) *Han & Dadey* (1976) and *Potter & Moore* (1977) but in agreement with *Lohrmann et al* (1974a) *van Oers & Zeijlemaker* (1977) *Lonai & McDevitt* (1977) and *Blomgren* (1977) Also in a recent study *Hansen et al* (1977) were able to demonstrate that these stimulatint T cells probably are contained within the Fc negative subpopulation

The role of monocytes in the human MLR has not been extensively studied though *Alter & Bach* (1970) *Twomey et al* (1970) and more recently *Blomgren* (1977) found that macrophages from both stimulator and responder donors ...

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... The monocytes used in this study which were cultured from Petri dishes showed very little – if any – stimulatory capacity at the concentration used in the experiments shown in Table 2 (data not shown) The addition of graded numbers of monocytes to MLR cultures (Fig 2) showed that beyond the optimal concentration monocytes inhibited the response This cannot be due simply to crowding in the culture because addition of irradiated lymphocytes from the responder donor did not show this effect

It can be speculated that soluble mediators are responsible for the inhibition of thymidine incorporation this phenomenon has been demonstrated for peritoneal exudate cells (*Fernbach et al* 1976) activated macrophages alone (*Nelson* 1973) and both normal and activated macrophages (*Calderon & Unanue* 1975) *Fernbach et al* (1976) also showed that inhibition of DNA synthesis in low to medium concentrations of monocytes could be due to competition of »cold« thymidine synthesized by the monocytes However that study also demonstrated that at higher concentrations of monocytes the MLR was truly inhibited Further support for an inhibitory role of monocytes in the human MLR comes from a study by *Laugther & Twomey* (1977) who showed that suppression of MLR responses equated with the presence of a cell rich in cytoplasmic esterase Likewise *Weiss & Fitch* (1977) showed that macrophages present in normal rat spleen preparations could account for the difficulty in generating cytotoxic T-cells in MLR s prepared with rat cells

Our data therefore suggest a dual role for monocytes in the human MLR in that they enhance the response at lower concentrations and inhibit it at high concentrations Whether these functions are exerted by the same cells or by separate subpopulations of monocytes or include the same or different mediators cannot be judged from our results

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LYMPHOCYTES FROM ADENOID VEGETATIONS PROLIFERATIVE RESPONSES *IN VITRO* AS COMPARED TO BLOOD LYMPHOCYTES

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Mogensen H H Meistrup Larsen K I & Andersen V Lymphocytes from adenoid vegetations Proliferative responses *in vitro* as compared to blood lymphocytes Acta path microbiol scand Sect C 87 197–202 1979

Thymidine incorporation by lymphocytes obtained from adenoids (AVL) and blood (PBL) were compared in 27 children undergoing adenoidectomy Optimal conditions as regards cell number and duration of culture were worked out The spontaneous thymidine incorporation was higher in PBL than in AVL In cultures stimulated by polyclonal activators or by PPD the responses of PBL were higher The dose response curves for PBL and AVL after stimulation with killed *H influenzae* were different PBL showed a higher response the optimal antigen concentration was lower for PBL and the responsiveness to suboptimal antigen concentrations was higher in PBL than in AVL

Key words Lymphocytes adenoid vegetations proliferative responses

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In mucosal defences against pathogenic microorganisms most attention has been paid to secretory antibodies Little information is available concerning the role of local cell mediated immunity

The aim of the present study was to investigate the proliferative responses *in vitro* of lymphocytes obtained from adenoid vegetations (AVL) and compare these to the responses of blood lymphocytes (PBL) The influence of cell number and duration of culture was established lymphocyte responsiveness to polyclonal activators was assessed and the responses to heat killed *Haemophilus influenzae (HI)* a common cause of upper respiratory tract infections were analyzed

MATERIAL AND METHODS

Subjects Fourteen girls and 13 boys aged 2–14 years (average 6 years) were studied The children were admitted to the Department of Otolaryngology Kommunehospitalet, for adenoidectomy because of recurrent upper respiratory infections, enlargement of the adenoid or a combination of both The children were in good health at the time of the investigation and none received any drug treatment

Operation and sampling Immediately after induction of anesthesia (with diazepam halothane suxamethonium chloride and atropine) 8–15 ml of blood were drawn in 10 ml of RPMI 1640 with heparin Within 1 hour the cells were isolated by gentle pressing of the tissue against a steel screen The lymphocyte transformation technique has been described in detail previously (3) Briefly mononuclear cells were isolated on a Ficoll Isopaque gradient washed 3 times and resuspended in RPMI 1640 containing 15 per cent pooled serum from healthy young non transfused male donors According to the results of cell titration experiments (see below) 10^5 cells were cultured per vial (round bottomed) in 500 µl medium at 37.5° C in 5 per cent CO_2 ^{14}C-thymidine was added 24 hours before termination and the incorporation was quantitated by liquid scintillation counting The results given are means of triplicate determinations after subtraction of the values obtained in unstimulated cultures A response in a stimulated culture is considered positive when it is more than 2.5 times the value of the corresponding unstimulated culture

Polyclonal activators Maximal stimulation of human blood lymphocytes was obtained with the following doses 50 µl of the 500 ml reconstituted stock solution of phytohaemagglutinin (PHA-P, Difco), 10 µl of the 25 ml reconstituted stock solution of pokeweed mitogen (PWM, Grand Island Biological Co), and 20 µg of Concanavalin A (Con-A, Pharmacia) For PHA, a dose-response determination was carried out in each experiment (see Results)

Microbial antigens Heat-killed (66° C, 30 min) *Haemophilus influenzae* was kindly prepared by Dr *Frits Orskov*, *Statens Seruminstitut*, Copenhagen, and employed in 10 concentrations, maximally 10^8 bacteria per culture with 3-fold dilutions Purified protein derivate (PPD), *Statens Seruminstitut*, was employed in 8 concentrations, maximally 5 µg per culture with 3-fold dilutions The maximal response is defined as the highest value obtained by any of the varying antigen doses

Statistical methods For comparison of lymphocyte transformation responses between AVL and PBL, the Wilcoxon rank sum test was employed The difference between *H influenzae* concentrations giving optimal stimulation of PBL and of AVL was evaluated by the binomial distribution test

RESULTS

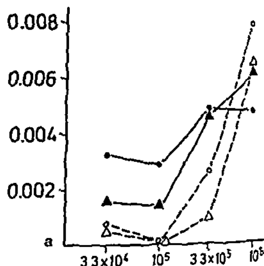
Influence of Cell Density

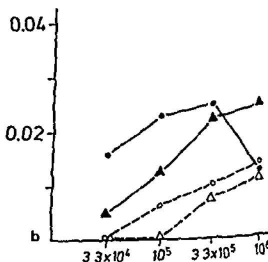
Lymphocytes were cultured at 4 different cell densities ($3\,3 \times 10^4$ to 10^6 in a volume of 500 µl) The results are shown in Fig 1 In unstimulated cultures, thymidine uptake was lower with AVL than with PBL except at the highest cell density tested When lymphocytes from two BCG-vaccinated persons were stimulated with PPD, thymidine uptake per cell increased with increasing cell density The values obtained for PBL were higher than for AVL After stimulation with *H influenzae* thymidine uptake peaked at cell density 10^5 or $3\,3 \times 10^5$ cells per culture

On the basis of these results, a cell density of 10^5 cells per 500 µl culture was chosen in subsequent experiments

Kinetics of the Response

Unstimulated cultures and cultures stimulated with PPD or *H influenzae* were harvested at varying intervals after initation of culture Experi-





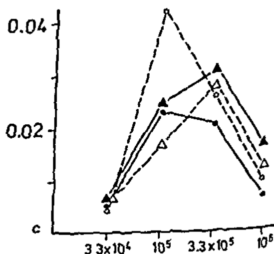
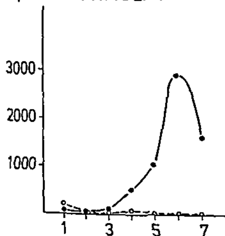
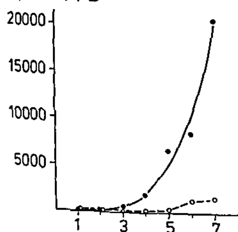


Fig 1 The effect of varying cell densities on thymidine incorporation, expressed as counts per minute per cell originally cultured The results obtained with adenoid [illegible] (open symbols) and blood cells (closed symbols) [illegible]

with 10^7 killed *H influenzae* [illegible] cultured for 5 days Note different scales of the ordinates.





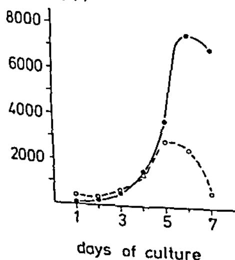


Fig 2 The effect of varying periods of culture on thymidine incorporation by lymphocytes from blood (●—●) and adenoid tissue (O—O) the donor was BCG vaccinated 10^5 cells were cultured for 1 to 7 days Note different scales of the ordinates

ments were carried out in 3 persons with similar results and a typical result is shown in Fig 2 In unstimulated cultures lymphocytes obtained from blood but not from adenoids started proliferation on the fourth day of culture with a peak after 6 days In PPD stimulated cultures of lymphocytes from BCG vaccinated donors the thymidine uptake by PBL increased rapidly after the third day of culture while a much smaller uptake by AVL was seen on the two last days of culture only Following *H influen ae* stimulation a peak in responses was obtained for both cell types For PBL on day 6 of culture at a high level for AVL one day earlier at a lower level

On the basis of these results a culture period of 5 days was employed in subsequent experiments

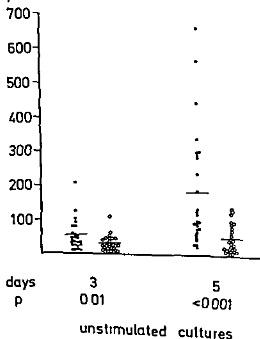


Fig 3 Thymidine uptake by unstimulated lymphocytes from blood (●) and adenoid tissue (O) after 3 and 5 days of culture (27 subjects) Lymphocytes obtained from adenoids showed significantly less incorporation than blood lymphocytes p-values are given

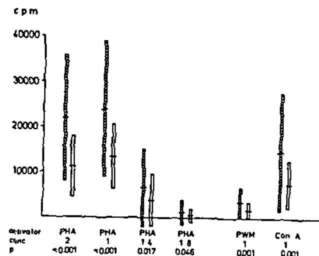


Fig 4 Thymidine uptake by lymphocytes after stimulation with PHA (4 concentrations), PWM and Con-A (mean values in 27 subjects, ± 2 SD) Lymphocytes obtained from adenoids (open columns) showed significantly smaller responses than blood lymphocytes (hatched columns), p values are given The optimal concentration for each stimulant (see Material and Methods) is given as 1

Unstimulated Cultures

Thymidine incorporation by unstimulated lymphocytes of the 27 subjects examined is shown in Fig 3 AVL showed lower activity than PBL Corresponding values for AVL and PBL in the individual patients are compared in Table 1

Polyclonal Activation

The responses to the polyclonal activators PHA, PWM and Con-A are shown in Fig 4 In all cases, AVL responded to these activators, the responses obtained were lower than those of PBL (p values are given in Fig 4) Dose titration was carried out for PHA 50 µl (see Material and Methods) induced maximal response in 19 of 25 samples of PBL and in all 25 samples of AVL The responses to suboptimal doses were similarly reduced in AVL and in PBL (Fig 4)

Corresponding values for AVL and PBL in the individual persons are compared in Table 1 It appears that in the large majority of persons examined, the response of PBL was higher than that of AVL.

Stimulation by H influenzae

A positive response was obtained in 26 of 27 subjects with AVL and in all 27 subjects with PBL. A typical pair of dose-response curves for PBL and AVL, using heat-killed *H influenzae* as stimulant is given in Fig 5 The mean of the maximal responses in 27 patients was 1871 c p m for PBL (range 293–4587 c p m) and 895 c p m for AVL (range 14-3542 c p m), this difference is statistically significant (p < 0 01) No correlation was found between age and the response of AVL or PBL The concentration inducing maximal thymidine incorporation varied for PBL between 4 1 × 10^5 and 10^8 bacteria per culture and for AVL between 1 2 × 10^6 and 3 3 × 10^7 bacteria per culture The most frequent optimum was at 0 37 × 10^7 bacteria for PBL and 1 1 × 10^7 for AVL. In 17 out of 27 cases, the optimal antigen concentration was higher for AVL than for PBL, in 7 cases there was no difference and only in 3 the optimal antigen concentration was lower for AVL. This difference (17 vs 3) is statistically significant (P = 0 5

TABLE 1 *Comparison of Thymidine Incorporation by Lymphocytes Obtained from Blood (PBL) and from Adenoids (AVL) The Polyclonal Activators Were Compared Employing the Optimal Concentration* H influenzae *and PPD by the Maximal Response*

	Thymidine incorporation higher in PBL (no of cases)	Thymidine incorporation higher in AVL (no of cases)	% of cases showing PBL > AVL
3 days of culture			
unstimulated	21	6	78
PHA	23	4	85
PWM	21	3	88
Con A	17	5	77
5 days of culture			
unstimulated	24	3	89
H influenzae	24	3	89
PPD	26	1	96

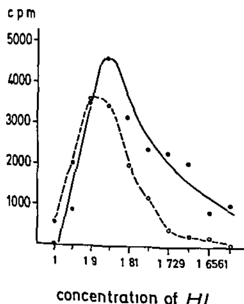


Fig 5 Thymidine incorporation by lymphocytes obtained from adenoids O and blood ● after stimulation with varying concentrations of killed *H influenzae (HI)* (1 is equal to 10^8 bacteria per culture)

significance 0 26%) Upon dilution of antigen responsiveness decreased more rapidly in AVL thus the response to 1/27 of the optimal antigen concentration expressed as per cent of maximal response was significantly lower for AVL than for PBL (p = 0 04)

In conclusion AVL show a smaller response to heat killed *H influenzae* than do PBL, they need a higher antigen concentration for maximal stimulation and their response disappears more rapidly upon dilution of antigen

DISCUSSION

In a recent study (2) we found a slightly decreased proportion of T lymphocytes and a markedly increased proportion of B lymphocytes in adenoid tissue as compared to blood Further, the proportion of esterase positive monocytes was about 15 per cent in cells prepared from blood and about 2 per cent in cell suspensions obtained from adenoid tissue

In the present study these cell preparations were employed to compare the proliferative responses *in vitro* of AVL and PBL. Qualitatively they were similar, but several quantitative differences were found An unexpected finding was that unstimulated cultures of lymphocytes, which are often supposed to reflect *in vivo* stimulation, showed lower thymidine incorporation by AVL than by PBL One possible explanation for this difference is that lymphocytes may leave the lymphoid tissue after activation (1) However, it cannot be ruled out that stimulated cells adhere more firmly to the stroma than unstimulated cells, so that they are more difficult to elute during preparation

The dose-response pattern to PHA was similar for the two types of cells However, the lymphocyte responsiveness to all three polyclonal activators studied was significantly lower in AVL than in PBL This is in contrast to the findings of *Rynnel Dagöö et al* (1977), who found similar DNA synthesis rates after stimulation of lymphocytes obtained from adenoids and blood Since the proportion of T lymphocytes is smaller in adenoids than in blood a lower thymidine incorporation, as found in the present study, would be expected in AVL after PHA and Con-A stimulation

Lymphocytes stimulated by heat killed *H influenzae* were studied sequentially in culture AVL showed a lower and earlier peak response than PBL. In similar studies of blood lymphocytes stimulated by PPD, cells from highly sensitized persons showed a higher and earlier appearing peak of thymidine incorporation (5) The finding in the present study of an early and low peak response in AVL may indicate different mechanisms of growth regulation in lymphocytes obtained from adenoids as compared to blood lymphocytes and not necessarily different degrees of sensitization

After 5 days of culture, the average peak response to *H influenzae* was twice as high in PBL as in AVL and appeared at a lower antigen concentration Further, the sensitivity to suboptimal antigen concentrations was greater in PBL than in AVL The optimal concentration varied with a factor 243 for PBL and 27 for AVL Thus, for both cell types it is necessary to use a range of concentrations in order to ensure that the maximal response is obtained

The causes of the differences in response between AVL and PBL are not clear The cells were washed three times before culture, so possible soluble factors adsorbed to the cell membrane *in vivo* must [illegible]

be investigated by the addition of purified monocytes such experiments are in progress in our laboratory Possible differences in regulatory mechanisms between AVL and PBL can be elucidated by examining the responses of autologous mixtures of these two cell populations

Our results indicate that adenoid vegetations contain cells capable of proliferation following mitogenic and antigenic stimulation This was found during early childhood as well *i e* the period when upper respiratory tract infections are frequent Placed at the beginning of the respiratory tract containing huge numbers of lymphoid cells the adenoid must be important in the first line of defence against microorganisms It will be of importance to establish whether the local cellular immune response to the antigens of a particular microorganism is related to the clinical susceptibility to infection

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Acta path microbiol scand Sect C, 87 203–211, 1979

CYTOLOGICAL EVENTS IN ALLO-STIMULATED LYMPHOCYTES TRIGGERED BY EXPOSURE TO STIMULATORY ALLOANTIGENS

II Changes in the Areal Density of Cytoplasmic Vacuoles and in the Subcellular Localization of Acid Phosphatase

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Brix Poulsen P & Nielsen L H Cytological events in allostimulated lymphocytes triggered by exposure to stimulatory alloantigens II Changes in the areal density of cytoplasmic vacuoles and in the subcellular localization of acid phosphatase Acta path microbiol scand Sect C, 87 203–211 1979

H 2b lymphocytes were sensitized against H 2d alloantigens by lymphocyte culture reaction and incubated with H 2d mastocytoma cells The interaction between lymphoid cells and mastocytoma cells was stopped by fixation with glutaraldehyde The areal density of the cytoplasmic vacuoles as well as the subcellular localization of acid phosphatase in lymphocytes were examined by electron microscopy Two populations of lymphocytes were observed small lymphocytes with heterochromatic nuclei and larger lymphocytes (lymphoblasts) with euchromatic nuclei Only the lymphoblasts showed change following interaction with target cells The vacuole area in percent of cytoplasmic area (vacuole areal density) of sensitized lymphoblasts increased during the first 30 minutes and from the third to fourth hour of interaction with target cells Acid phosphatase staining was observed in the Golgi apparatus of the lymphoblasts after 30 minutes of interaction Multivesicular bodies showed acid phosphatase staining within 20 minutes of interaction with target cells After 20 minutes of interaction, phagosomes containing myelin figures were formed These phagosomes also showed acid phosphatase staining and during the next hours of interaction their number increased over the number of multivesicular bodies

Key words Lymphoid cells Golgi apparatus lysosome vacuole

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Received 28 ix 78 Accepted 16 xii 78

Lysosomes which contain hydrolytic enzymes are [illegible] lar [illegible] [illegible] hydrolases have been demonstrated in lymphocytes stimulated with phytohemagglutinin (PHA) (Hirschhorn et al 1967 Cohen et al 1973, and [illegible] [illegible] in previous studies (*Brix Poulsen et al* 1975 and *Brix Poulsen & Guttler* 1975), we have found positive staining for lysosomal enzymes during interaction with target cells in lymphocytes stimulated against the H-2 alloantigens of the target cells, but during interaction between the same target cells and lymphocytes stimulated against H-2 alloantigens different from the alloantigens of the target cells there was no staining for lysosomal enzymes in the lymphocytes

Zagury et al (1975) examined the ultrastructure of isolated killer lymphocytes and the lysis of

Our results indicate that adenoid vegetations contain cells capable of proliferation following mitogenic and antigenic stimulation This was found during early childhood as well *i e* the period when upper respiratory tract infections are frequent Placed at the beginning of the respiratory tract containing huge numbers of lymphoid cells the adenoid must be important in the first line of defence against microorganisms It will be of importance to establish whether the local cellular immune response to the antigens of a particular microorganism is related to the clinical susceptibility to infection

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II Changes in the Areal Density of Cytoplasmic Vacuoles and in the Subcellular Localization of Acid Phosphatase

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Lysosomes which contain hydrolytic enzymes are important in destroying cellular and extracellular components (*de Duve & Wattiaux* 1966 *Woodin & Wienecke* 1964 and *Faes* 1968) Acid hydrolases have been demonstrated in lymphocytes stimulated with phytohemagglutinin (PHA) (*Hirschhorn et al* 1967 *Cohen et al* 1973 and *Bitterfeld* 1971) and after stimulation with mixed lymphocyte reaction (MLR) (*Hirschhorn et al* 1967) and tumor allo-graft immunization (*Brix Poulsen et al* 1975 and *Brix Poulsen & Guttler* 1975) In previous studies (*Brix Poulsen et al* 1975 and *Brix Poulsen & Guttler* 1975) we have found positive staining for lysosomal enzymes during interaction with target cells in lymphocytes stimulated against the H 2 alloantigens of the target cells but during interaction between the same target cells and lymphocytes stimulated against H 2 alloantigens different from the alloantigens of the target cells there was no staining for lysosomal enzymes in the lymphocytes

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Fig 1 Vesicular structures in the cytoplasm of a lymphocyte The vesicles between the arrows are smaller than 0 2 μ (the largest of them is exactly 0 2 μ) 17 160 X

RESULTS

The cell population after MLR-stimulation consisted of two types of lymphoid cells a small type with heterochromatic nucleus and a larger type with euchromatic nucleus The latter type of the lymphocytes was the only one which exhibited changes following interaction with target cells This cell resembles a blast cell and will be referred to as such in the following section

The Areal Density of the Cytoplasmic Vacuoles

The blast cells showed an increase in vacuole areal density (the vacuole area in percent of cytoplasmic area) from 1 7% to 11 0%, within the first 30 minutes of interaction with target cells In the next thirty minutes this increase was followed by a decrease to 6 6% From one to three hours of interaction with target cells the vacuole areal density was nearly constant From three to four hours of interaction the vacuole areal density of the blast cells increased to 14 7% (Table 1)

Considering the increase in cytoplasmic area of blast cells during the first 3 hours of interaction the net formation of vacoules was greatest within the first twenty minutes of interaction whereas the formation was equal to the destruction between one and two hours of interaction (Table 1)

TABLE 1 *Change in Formation and Destruction of Cytoplasmic Vacuoles in Lymphocytes in Relation to Time of Interaction with Target Cell*

Time of incubation (hours)	Vacuole areal density[a] in blast cells (%)	Probability (p)	Vacuole volume per cell (μ^3/cell)	Vacuole ratio [b]	Vacuole areal density[a] in control cells (%)	Probability (p)
0	1 7		1 4		1 5	
1/3	9 2	p<0 01	30 5	21 8	1 8	p>0 05
1/2	11 0	p<0 01	49 0	1 6	2 8	p>0 05
1	6 6	p<0 05	36 4	0 7	3 4	p>0 05
2	5 7	p<0 05	37 3	1 0	3 2	p>0 05
3	6 0	p<0 05	82 0	2 2	2 8	p>0 05
4	14 7	p<0 01	125 5	1 5	2 8	p>0 05

[a] Vacuole area in percent of cytoplasmic area

[b] Value obtained when the observed volume (μ^3) of vacuoles in blast cells is divided by the expected volume assuming there was no formation and destruction of vacuoles from the preceding observation time

Values > 1 indicates formation is greater than destruction

< 1 indicates formation is smaller than destruction

1 indicates formation is equal with destruction

conjugated target cells They *observed that the* cytotoxic lymphocytes were small cells with an intended nucleus which was *poor in* peripheral chromatin The cytoplasm *contained* one membrane-bound lysosome like granules and the Golgi apparatus was well developed Moreover *Matter & Simpson* (1976) observed that the biggest cell observed in cultures with strong cytotoxic activity was a blast cell of 20 μ diameter with a huge nucleolus and an enormous number of *polyribosomes in the cytoplasm This cell type was observed* most frequently around day 2–4 of the cultures The blast cells *often showed* well developed uropods containing numerous vesicles (*Matter & Simpson* 1976)

In a previous paper (*Brix Poulsen* 1979) it was shown that the concentration of the *granulated* endoplasmic reticulum as well as the Golgi apparatus in lymphocytes stimulated by MLR was highest at the onset of interaction with target cells This showed *that a readiness state* for synthesis of vacuoles and lysosomes was present in the lymphocytes after MLR stimulation Despite the knowledge of the turnover of membranes and *macromolecules on the surface of lymphocytes* (*Pasternak & Friedrichs* 1970 and *Hayden et al* 1970) little is known about the kinetics of the cytoplasmic vacuoles and lysosomes in stimulated lymphocytes The aim of the present study was to examine the kinetics of vacuole formation and especially lysosome *formation in MLR stimulated lymphocytes* during interaction with target cells

MATERIAL AND METHODS

Animals Mice of inbred strains DBA/2J (H 2^d) and *C57Bl/6J (H 2^b) were obtained from* GI Bomholtgaard Laven Jutland F_1 hybrids of C57Bl/6J X DBA/2J were bred at the Institute of Medical Anatomy Copenhagen

Cells Effector cells C57Bl/6J lymphocytes from spleen and lymph nodes *Stimulator cells* F_1 [C57Bl/6J X DBA/2J] lymphocytes from spleen and lymph nodes *Control cells* C57Bl/6J lymphocytes from spleen and lymph nodes were kept *in vitro* under the same conditions as effector cells concerning medium and time in vitro *Target cells* The tumour P 815 X 2 mastocytoma *of* H 2^d origin was kindly donated by Dr Bent Rubin Statens Seruminstitut, Copenhagen

Cell preparation Spleen and lymph nodes were obtained aseptically and homogenized by grinding [illegible] t 400 The cells were [illegible] fered saline pH 7 2 [illegible] 3S and stored for 15 [illegible] bris to settle Then the cells were spun down and resuspended in 156 6 mM NH_4Cl in distilled water at 4° C for 10 minutes which disrupted the erythrocytes After three washes the cells were resuspended in culture media and counted

Cultivation medium 500 ml RMPI 1640 with 25 mM Hepes buffer (240SI Gibco) 25 ml (5%) *foetal calf* serum (629 Gibco) 5 ml (1%) L glutamine 200 mM (503 Gibco) 125 000 i u Penicillin (Novo) 0 05 g Streptomycinsulfate (Novo) and 5 × 10 mM 2 mercaptoethanol

Acid phosphatase stain A Stock solutions for Gomori stain

a) 0 02M sodium – β – glycerophosphate (Merck 4168) in 0 04M trismaleate (Sigma 7–9) (pH 5 2)

b) 0 006M lead nitrate (Merck 7398) in distilled water

B Incubation medium for Gomori stain

25 ml of stock solution »a« plus 20 ml of stock »b« plus 5 ml distilled water pH adjusted to 5 2 with 1 N NaOH

Stimulation of lymphocytes 30×10^6 *stimulator cells and* 30×10^6 effector cells were mixed in Falcon tissue culture flask No 3018 with 20 ml cultivation medium *and stored* for 4 days at 37° C in an atmosphere containing 5% CO_2

Cytolysis fixation and embedding Four days after initiating the stimulation the *lymphocyte* suspensions were counted and mixed 10 1 with tumour cells

After zero 20 and 30 minutes and after 1 2 3 and 4 hours the cell suspensions were spun down at 400 g for 6 minutes and the cytolysis was stopped by fixation in 3% glutaraldehyde in 0 1 M *phosphate* buffer (pH 7 2) at 4° C for 2 hours and washed for 12 hours in 0 1 M phosphate buffer (pH 7 2) 4° C

The cell pellets were then embedded in agar sectioned *at 40–50 μ incubated in the Gomori stain* as described by *Barka & Anderson* (1962) *for 90 minutes at* 37° C, and washed 3 × 15 minutes in 0 04 M trismaleate Post fixation was performed by 2% OsO_4 in 0 1 M phosphate buffer (pH *7 2) for 2* hours at 4° C The sections were then washed in 0 1 M phosphate buffer (pH 7 2) at 4° C and three times for 30 minutes at 4° C in distilled water Subsequently the sections were placed in 0 5 M uranyl acetate in distilled water for 12 hours at 4° C and finally washed for 20 minutes at 4° C in distilled water The sections were then dehydrated in increasing concentrations of ethanol and embedded in Epon

Electron microscopy Silver to gray thin sections were cut on an LKB Ultrotome and stained with uranyl acetate and lead citrate The cells were then examined and photographed using a *Philips EM 300 electron microscope* at an accelerating voltage of 60 KV

Quantitation and statistics The lymphocytes cytoplasmic area and the area of the cytoplasmic vacuoles were measured and calculated stereologically with a Leitz A S M image system

[illegible] was used for the [illegible] of [illegible] (in each observation period 40 lymphoblasts were counted along with an equal number of control cells Vesicular structures smaller than approxima[illegible] measured (Fig 1)

The control cells showed no significant change in the areal density of the cytoplasmic vacuoles within the four hour period of observation (Table 1)

The Appearance of Acid Phosphatase in the Golgi Apparatus

Acid phosphatase was not observed in the Golgi apparatus of the blast cells before 30 minutes of interaction with the target cell whereas afterwards the staining was seen in about 80% of the blast cells (Fig 2-5)

Control cells did not exhibit acid phosphatase in the Golgi apparatus before three hours of interaction with target cells At this time it was seen in 10% of the cells

The Appearance of Acid Phosphatase in cytoplasmic Vacuoles

The following types of acid phosphatase positive vacuoles (lysosomes) appeared in blast cells during interaction with target cells 1) lysosomes containing small vesicles — multivesicular bodies (MVB) 2) lysosomes containing myelin figures — phagosomes and 3) lysosomes containing residuals — residual bodies

Acid phosphatase in vacuoles of blast cells was not found at the onset of interaction with target cells During the first twenty minutes of interaction with target cells acid phosphatase was found in the matrix of about 50% of the MVB (Fig 6) After thirty minutes phagosomes developed (Fig 7 and 8) and after two hours of interaction with the target cells residual bodies were observed (Fig 5a and 8)

After one hour lysosomes constituted 10% of the vacuole system (Table 2), most of them phagoso-



Fig 6 Multivesicular bodies (MVB) in a blast cell after 1 hour of interaction with target cells One of the MVB shows lead precipitation indicating acid phosphatase 56 600 X

TABLE 2 *Changes in the Amount of Lysosomes in Blast Cells in Relation to Time of Interaction with Target Cells*

Time of incubation (hours)	The areal density of lysosymes a) (%)	Probability (p)	Lysosomes in percent of vacuoles (%)	Lysosome volume per cell (µ³/cell)	Lysosome ratio b)
0	0 0		0 0	0 0	
		p<0 01			
1/3	0 2		3	0 9	>1
		p<0 05			
1/2	0 7		6	2 9	3 2
		n s			
1	0 7		10	3 6	1 2
		n s			
2	0 7		12	4 9	1 4
		p<0 05			
3	1 3		22	18 0	3 7
		p<0 01			
4	3 1		21	26 4	1 5

n s Not significant
a) The areal density = the area in perce[illegible]
b) [illegible]











Figs 2–4 Golgi regions displaying lead precipitation indicating acid phosphatase in blast cells after 2 hours of interaction with target cells Fig 2 and 3 20 000 X Fig 4 37 000 X

Figs 5a–5b Phagosomes with myelin figures and Golgi regions displaying lead precipitation indicating acid phosphatase in blast cells after 3 hours of interaction with target cells The phagosome in »a« contains a residual body (arrow) a 26 000 X b 42,000 X

and did not decrease throughout the four hour observation period The maximum concentration of the granulated endoplasmic reticulum in the lymphocytes was found at the onset of interaction with target cells

The fast increase of the vacuole areal density during the first thirty minutes of interaction (Fig 2) may be explained by the increase of the first Golgi »generation« during the first twenty minutes and by the high concentration of granulated endoplasmic reticulum The decrease in vacuole areal density between thirty minutes to one hour of interaction may be caused by the time lapse between the decrease of the first »generation« and the increase of the second »generation« of the Golgi apparatus

Thus, there seems to be two main periods of vacuole formation one from zero to thirty minutes, and one from the second to the third hour of lymphocyte target cell interaction During these periods the diameter of the lymphocytes increases quickly (*Brix Poulsen* 1979) This concurrent increase in the vacuole system and the cell diameter can be explained by a conversion of vacuole membranes to plasma membrane, when vacuoles fuse with the cytoplasmic membrane and release their contents on the outside of the cell This would be in agreement with the observation by *Hawkins* (1971) and *Henson* (1971) who showed a release of lysosomal enzymes on the outside of lymphocytes encountering immune complexes which have been dispersed along a non phagocytosable surface such as a collagen membrane or a Millipore filter A conversion of vacuole membranes (and not especially lysosome membranes) to cytoplasma membrane would permit structures on the inner surface of the vacuoles to be present on the outer surface of the cytoplasmic membrane This might provide structures on the cell surface which are necessary for the recognition and contact with the target cell in the recognition (adhesion) phase of cytolysis and/or in the lethal hit phase as described by *Golstein & Smith* (1977)

In previous papers (*Sanderson & Glauert* (1977) and *Brix Poulsen* (1979) using electron microscopy a contact between stimulated T cells and target cells was described The cell membranes were clearly separated but the two cells were in close continuous contact over considerable distances Later on projections from the T cells penetrated into the target cell

Light microscopic examination of stimulated T-lymphocytes has revealed an increased staining of acid phosphatase beta glucuronidase and esterase in the plast cell population (*Brix Poulsen et al* 1975 and *Brix Poulsen & Guttler* 1975) and *Biberfeld* (1971) observed acid phosphatase activity in the Golgi apparatus of PHA stimulated lymphoid cells Electron microscopy used in the present study has extended these observations The appearance of acid phosphatase in MLR-stimulated lymphocytes began after 20 minutes of interaction with the target cells and at that time acid phosphatase appeared in MVB After thirty minutes, acid phosphatase was also observed in the Golgi apparatus and the phagosomes

The myelin figures in the phagosomes may indicate that the enzyme was engaged in destruction of membrane structures There may be various sources providing the membranes inside the phagosomes Target cell membranes or destruction of the lymphocytes own membrane structures

It remains uncertain whether lysosome forma-

contents from lysosomes and other vacuoles in the lymphocyte into the intercellular cleft between the lymphocyte and the target cell in the same manner as the release of enzymes by macrophages as described by *Hibbs* (1974) and *Temple et al* (1973) However a release of cytotoxic enzyme(s) from a killer cell has to be »polarized« toward the target cell because it has been shown that the killer cells as well as bystander cells are unharmed during the target cell lysis (*Cerottini & Brunner* 1974, *Martz* 1976b *Sanderson* 1976, *Zagury et al* 1975)

Another mechanism could be the release of hydrolytic enzymes directly from the lysosomes into target cells This has been shown to take place when phagocytic cells surrounding a *Cryptococcus neoformans* release hydrolytic enzymes into the encircled yeast (*Kalina et al* 1971) A third mechanism could be destruction of target cell membranes inside lysosomes of the lymphocyte, which could explain the presence of myelin figures in the phagosomes The latter mechanism would then be a secondary destruction of target cells, because it would require a preceding destruction of the target cells »in situ« As »the lethal hit« seems to occur within the first 10 minutes of interaction (*Berke et al* 1972 and *Martz* 1976a) the present finding might be secondary to target cell lysis e g initial events occuring in lymphocytes attached to dying target cells

Two unclarified aspects of our experiments are that it is not possible to determine 1) which of the lymphocytes are the cytotoxic ones and which ones



Fig 7 Phagosomes with myelin figures present in a blast cell after 30 minutes of interaction with target cells The phagosome to the right displays lead precipitation indicating acid phosphatase 60 000 X

Fig 8 Phagosomes with residual bodies present in a blast cell after two hours of interaction with target cells Both phagosomes show lead precipitation indicating acid phosphatase 37 000 X

mes, and after three hours of interaction there was an increase mostly of phagosomes in the lysosomes to 22 % of the vacuole system From the third to the fourth hour of interaction there was an increase in residual bodies, but there was no increase in [illegible] the

The formation of acid phosphatase positive vacuoles (lysosomes) in the blast cells was greater than the destruction during the observation time and considering the concurrent increase in cell size, the formation was greatest from zero to thirty minutes, and from hour 2 to 3 (Table 2)

Control cells did not exhibit acid phosphatase in vacuoles before two hours of interaction, but then acid phosphatase positive vacuoles were found in 16 % of the cells, and after four hours it was found in 28 % of the cells The areal density of lysosomes in the control cells containing lysosomes was 0 3 % after two hours of interaction with target cells, and 0 5 % after 4 hours of interaction

DISCUSSION

In our experiments the stimulator cells were obtaining from an F_1 hybrid raised between two histoallogenic strains, one of the responder cell origin (H-2[b]), and the other isogenic with the mastocytoma origin (H-2[d])

It has been shown (*Wilson* 1967, *Wilson et al* 1967, *Harrison et al* 1968 and *Clancy & Rieke* 1969), that despite active proliferation of parental cells in parent-F_1 mixtures, few, if any, F_1 cells divide as a consequence of the mixture Furthermore, in preceding experiments (unpublished) we have shown that after 4 days of stimulation, MLR-cultures, in which stimulator cells from F_1 hybrids have been treated with mitomycin, did not contain fewer blast cells than MLR-cultures in which the stimulator cells were untreated MLR-cultures in which the reponder cells were treated with mitomycin did not contain blast cells after 4 days The MLR-stimulation used in our experiments may thus be regarded as a»one-way« stimulation of the parental cells by the F_1 cells

In previous studies (*Elves et al* 1964, *Johnson & Roberts* 1964, and *Parker et al* 1965) it has been shown, that lymphocytes stimulated by phytohemagglutinin (PHA) develop smooth as well as coated vesicles and electron opaque lysosome-like bodies Stimulation of lymphoid cells using PHA is non specific as opposed to the MLR-stimulation in which the lymphocytes are specifically sensitized against alloantigens

Some of the prerequisites for the formation of vacuoles and thereby lysosomes in the lymphocytes during interaction with target cells have been discussed in a previous publication (*BrixPoulsen* 1979) These include a readiness of the granulated endoplasmic reticulum and the Golgi apparatus

The same study suggested two »generations« of the Golgi apparatus in the lymphoblasts (*Brix Poulsen* 1979) The first one increased from the onset of interaction, but after twenty minutes of interaction this »generation« of the Golgi apparatus decreased quickly The second »generation« increased after one hour of interaction with target cells,

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The vacuoles in the blast cells can be formed not only from the Golgi apparatus but also from the cytoplasmic membrane, if the cell takes up material from the outside Investigations of whether the blast cell takes up material from the outside during the four hour observation period are in progress

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COMPLEMENT ACTIVATION BY PNEUMOCOCCI ASSOCIATED WITH ACUTE OTITIS MEDIA

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Prellner K Complement activation by pneumococci associated with acute otitis media Acta path microbiol scand Sect. C 87 213-216 1979

Pneumococci (types I III VI XIV XVIII XIX and XXIII) associated with acute otitis media were shown to activate complement in normal human serum by the classical as well as by the alternative pathway In serum incubated with pneumococci classical pathway activation was demonstrated by decreased C4 values and the appearance of C1r-C1s-C1 IA complexes Pneumococci caused C3 conversion in C2-deficient serum and in serum chelated with Mg++ EGTA showing activation of the alternative pathway without participation of the C42 convertase Complement activation was more efficient when both pathways were intact This was evident from a more pronounced C3 conversion and a greater reduction of the values for properdin and factor B in non-chelated serum as compared to Mg++ EGTA chelated serum

Key words Complement activation pneumococci acute otitis media

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Relapsing acute otitis media in children most often caused by pneumococci (11) is a serious clinical problem The factors responsible for the relapses are not known

It is well documented that C3b plays a major role in the opsonisation of pneumococci by normal human serum (9 20) C3b can be generated by classical pathway activation initiated by antigen antibody complexes or by activation of the alternative pathway

Immunoglobulin levels are reported to be within normal levels in children with acute otitis media (1) Prophylactic trials with polyvalent pneumococcal polysaccharide vaccines have shown that the antibody response to some capsular types was suboptimal in children younger than two years of age (6) Aberration of the complement system has been reported in children with relapsing otitis media due to pneumococci (7) Thus C1q levels were depressed in 60 per cent of patients with relapsing otitis media in combination with normal or elevated levels of C1r and C1s Increased amounts of complexes composed of C1 subcomponents C1r C1s and C1r-C1s C1 IA were found (7) Thus opsonisation dependent on activation of the classical pathway might be impaired

The capacity of different pneumococcal types to activate complement has been reported by *Fine* (3) The persent study is concerned with the activation of complement by the pneumococcal types commonly present in relapsing acute otitis media (11)

MATERIALS AND METHODS

Normal human sera from three healthy donors and serum from a homozygotic C2 deficient adult were collected and frozen in aliquots at −80° C within 3 hours of sampling Specific pneumococcal polysaccharide antibodies to type I III VI XIV XVIII XIX and XXIII assessed by immunofluorescence (22) were present in low titers in the sera

Ethylenediamine tetracetic acid (EDTA) in a concentration of 20 mmol/l was used to block the classical and the alternative pathways

Streptococcus pneumoniae strains of serotypes III, VI XIV, XVIII, XIX, and XXIII were available from the department Serotype I, four strains, was supplied by Statens Seruminstitut, Copenhagen, Denmark Pneumococci were cultivated for 18 hours in Todd-Hewitt broth washed three times and suspended in phosphate buffered saline (0 12 M NaCl, 0 03 M phosphate) pH 7 2 The suspensions were adjusted to approximately 3×10^9 organisms per ml by density determinations at 540 nm In some experiments bacteria killed by boiling for 10 minutes were used

Treatment of sera with pneumococci Incubation was carried out at 37° C for 60 minutes using viable or heat-killed pneumococci at approximately 5×10^8 organisms per ml serum Before complement analysis, the bacteria were removed by centrifugation Normal serum Mg⁺EGTA chelated serum and C2 deficient serum treated at 37° C for 60 minutes and EDTA chelated serum incubated with pneumococci were used as controls

Hemolytic complement activity was determined according to *Kabat & Mayer* (10) and expressed as per cent of hemolytic activity of the relevant control serum Mg^{++} EGTA chelated sera were recalcified (25 mmol Ca^{++} per ml) before analysis

Quantitations of complement components The electroimmunoassay (14, 17, 21) was employed for determinations of C1q C1s, C3, C4, factor B and properdin

C1 subcomponents complexes Complexes of C1r-C1s and complexes of C$\bar{1}$r C$\bar{1}$s-C$\bar{1}$ IA were studied by crossed immunoelectrophoresis (13) C$\bar{1}$r C$\bar{1}$s-C$\bar{1}$IA complexes were measured immunochemically according to *Laurell et al* (16) Pooled normal serum treated with heat aggregated IgG with all C1s antigen present in the C$\bar{1}$r-C$\bar{1}$s C$\bar{1}$ IA complex was used as a reference On double determinations the time to time variation was 1 4 per cent (SD) (16)

C3 conversion was assessed by crossed immunoelectrophoresis (4) and evaluated by planimetry The proportion of converted C3, mainly C3c was expressed as a percentage of the total area outlined by precipitate In each assay, the C3 conversion was given as a percentage after correction for the C3 conversion present in the appropriate control

Statistical significance was assessed by t test for paired data

RESULTS

Complement components levels Incubation of normal serum with pneumococci of the serotypes studied did not change the levels of C1q C1s or C3 C4 and factor B values were slightly reduced in non-chelated sera after incuation with the pneumococci but not in sera chelated with Mg^{++} EGTA After incubation with pneumococci the concentration of properdin was low in non-chelated as well as in chelated sera (Table 1) Also in C2-deficient serum the properdin levels fell markedly on incubation with pneumococci In the non-chelated sera the properdin levels were significantly lower than in the Mg^{++} EGTA chelated sera ($p < 0.025$) The values of the complement factors did not differ in experiments using viable or heat-killed bacteria

All pneumococcal types activated complement which resulted in low residual hemolytic activity After incubation with pneumococci the residual hemolytic complement activity was reduced to less than 5 per cent of the original value in non-chelated serum and 10–15 per cent in Mg^{++} EGTA chelated serum

C1 subcomponent complexes Quantitation of C$\bar{1}$r-C$\bar{1}$s-C$\bar{1}$ IA complexes of the normal serum

TABLE 1 *Levels of C4 Factor B and Properdin in Normal Non chelated Serum and in Mg^{++} EGTA Chelated Serum after Incubation with Different Pneumococcal Serotypes*

Pneumococcal serotypes	C4		Factor B		Properdin	
	non chealted serum	Mg^{++} EGTA serum	non chelated serum	Mg^{++} EGTA serum	non-chelated serum	Mg^{++} EGTA serum
I	78	94	93	101	55	63
III	70	99	82	101	31	55
VI	67	89	82	109	48	55
XIV	63	103	76	109	39	55
XVIII	56	99	76	115	55	63
XIX	67	109	70	115	35	50
XXIII	70	99	76	124	48	63

Values given as percentage of non incubated sera

TABLE 2 *Per Cent of C3 Conversion Caused by Incubation with Pneumococci of Different Serotypes*

Pneumococcal serotype	Normal non-chelated serum	Mg + + EGTA chelated serum	C2-deficient serum
I	48	41	24
III	60	42	24
VI	53	42	25
XIV	52	41	24
XVIII	53	49	24
XIX	60	41	24
XXIII	54	42	23

The values were obtained by planimetric evaluation See Materials and Methods

used as control gave a value of 18 per cent of the reference serum (16)

On incubation of serum with pneumococci of different types the C1r-C1s-C1 IA complexes increased to values ranging between 30 per cent (type I) and 45 per cent (type XIX) indicating C1 activation (15) No C1r-C1s complexes were observed

C3 conversion All the pneumococcal types gave rise to C3 conversion in non-chelated Mg + + EGTA chelated and C2 deficient serum The C3 conversion was more pronounced in non-chelated serum as compared to Mg + + EGTA and C2 deficient serum (Table 2)

DISCUSSION

The present investigation showed that serotypes of *Streptococcus pneumoniae* most often found in acute otitis media (I III VI XIV XVIII XIX and XXIII) gave rise to C3 conversion in serum chelated with Mg + EGTA and in C2-deficient serum These findings indicate that the strains were capable to activate the al...

activation mainly by other pneumococcal types than those pertinent to relapsing otitis media His data concerning type III and XIV are in agreement with those found in this study On the other hand our results gave evidence of alternative pathway activation also by type I pneumococci (four strains tested)

The presence in serum of complexes composed of C1r C1s and C1 inactivator protein signifies activation of C1 (15) The f...

way activation which was also supported by the decreased C4 levels After treatment with pneumococci the residual hemolytic capacity diminished in non-chelated and in recalcified Mg + + EGTA chelated serum indicating marked C activation and consumption by the pneumococci

Quantitative estimation of factor B gave decreased values in the experiments with non-chelated serum only and probably indicates additional generation of C3 convertases when both pathways were intact It has been shown earlier that $\overline{C42}$ of the classical pathway by conversion of C3 to C3b is capable of initiating the C3b feed back cycle and thus activate factor B (8 18)

Pneumococci induced a marked decrease of properdin in C2-deficient serum, in non-chelated and in Mg + + EGTA chelated serum Reduction of properdin was most marked in non-chelated serum (Table 1) which might be explained by the amplification of the C3b feed back cycle by the $\overline{C42}$ convertase *McLean et al* (19) have shown that in normal human serum the concentration of properdin was decreased following incubation with antigen antibody complexes inulin or zymosan In C2-deficient serum reduced properdin values were obtained only with zymosan Thus the effect of pneumococci on properdin levels seems to be analogous to the effect ov zymosan

Heat killed bacteria were as potent as viable organisms in activating the complement system This finding indicates that complement activation was most probably not induced by bacterial enzymes

Complement activation by the classical pathway may be due to antigen antibody complexes The serum used contained small amounts of antibodies to capsular pneumococcal antigens Search for antibodies to other antigens as C polysaccharide (CPS) or pneumolysin was not done A complex of CPS and C reactive protein has been reported to activate both the classical and the alternative pathway (2 12) CRP-CPS complexes are probably

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Values given as percentage of non incubated sera

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ELECTRON MICROSCOPY OF TREPONEMES SUBJECTED TO THE *TREPONEMA PALLIDUM* IMMOBILIZATION (TPI) TEST

I Comparison of Immunoimmobilized Cells and Control Cells

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Hovind Hougen K Nielsen H Aa & Birch Andersen A Electron microscopy of treponemes subjected to the *Treponema pallidum* immobilization (TPI) test I Comparison of immunoimmobilized cells and control cells Acta path microbiol scand Sect C 87 217–222 1979

The ultrastructure of cells of *T pallidum* Nichols subjected to the TPI test was studied in negatively stained specimens Cells incubated in basal medium to which was added either human syphilis serum or heated guineapig serum (GPS) showed a normal morphology This was also the case for cells incubated with basal medium to which was added either human syphilis serum and heated GPS or normal human serum and unheated GPS By dark field microscopy cells obtained from these different incubation mixtures were found to be motile In contrast cells incubated in basal medium to which was added human syphilis serum and unheated GPS were all immobilized and in the electron microscope they presented a morphology strikingly different from that of normal cells The immunoimmobilized cells were swollen and their surface was completely covered with a layer of fuzzy material The nature of this material and its possible role in rendering the treponemes immobile is discussed

Key words *Treponema pallidum* *Treponema pallidum* immobilization (TPI) test electron microscopy immunoimmobilization

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The *Treponema pallidum* immobilization (TPI) test (6) is considered to be the most specific

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microscopy usually after 18 hours of incubation and a serum is regarded as containing specific antibodies when more than 20 per cent of the trepone[illegible]

present work was undertaken to study whether morphological differences between motile and immobile treponemes could be observed on cells incubated as specified for the TPI test when the cells were negatively stained and examined in the electron microscope

MATERIAL AND METHODS

Cells of *T pallidum* Nichols were obtained from rabb[illegible]

not responsible for complement activation observed in our experiments since the CRP level in the serum used was low (<12 mg per l)

Activation of the alternative pathway by pneumococcal cell wall preparations which contain teichoic acids and peptidoglycan have been shown by *Winkelstein & Tomasz* (23) One or both of these substances may be the pneumococcal constituent responsible for activating the alternative route (5, 23)

The pneumococcal types studied by us were capable of activating complement by both classical and alternative pathways and the conversion of C3 was most pronounced when both pathways were functional Earlier studies (7) showed impairment of the classical pathway in relapsing pneumococcal otitis media It is possible that generation of C3b by the alternative pathway alone might not be sufficient for optimal opsonisation and elimination of the pneumococci and therefore be a contributing factor to relapses observed in acute otitis media

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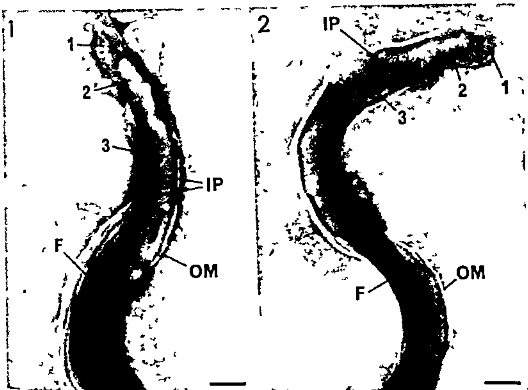
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All figures show cells of *T pallidum* Nichols subjected to the TPI test. The material was negatively stained with 1 per cent ammonium molybdate pH 7 2 The bar on each micrograph represents 100 nm and GPS in the legends means guinea pig serum

Figs 1 2 Cells of *T pallidum* incubated in basal medium with addition of normal human serum and unheated GPS (Fig 1) or human syphilis serum and heated GPS (Fig 2) The three zones normally present at the ends of the organism (see text) are clearly shown The outer membrane (OM) tightly envelops the cytoplasmic body of the cell IP denotes the insertion points for the flagella (F)

width of these immunoimmobilized cells was 0 21 µm whereas that of motile cells was 0 12 µm The width of the cytoplasmic body was identical for motile and immunoimmobilized cells but the zone 2 part of the cytoplasmic body was difficult to resolve on the majority of the immunoimmobilized cells (Figs 3 and 4) The flagella of the swollen treponemes as compared to those on motile cells were loosely wound around the cytoplasmic body of the cell (Figs 3 and 5) No flagella were ever seen to pierce or to protrude from the outer cell membrane On a few cells the outer membrane presented balloon like evaginations (Fig 5) thus emphasizing the loose texture of the membrane of the immuno immobilized treponemes

series of experiments were carried out The first was designed to investigate whether the swelling occurred continuously and progressively during the entire incubation period while the second was designed to study if swelling could be demonstrated on cells obtained from reaction mixtures in which the amounts of antibody were too low for the immunoimmobilization to proceed to 100 per cent during 8 hours of incubation. In the time dependence experiments only about 60 per cent of the treponemes incubated in plain basal medium for 6 8 and 12 hours were motile (Table 1) However all cells showed a morphology identical with that of normal cells Except for cells incubated with Cop 111 plus unheated GPS cells obtained from all the other tubes were also morphologically normal irrespective of the duration of incubation Treponemes incubated with Cop 111 plus unheated GPS showed a normal morphology after 0 and 4 hours of incubation while after 6 hours a little less than

heated at 56° C for 30 minutes The human sera used were Cop 111, which was obtained from a syphilitic patient (7) and Np which was a pool of sera obtained from healthy blood donors with no previous or present history of syphilis All sera in the Np pool were non reactive in the syphilis serology tests The human sera were inactivated for 30 minutes at 56° C before use After a suitable incubation time the results were read by dark field microscopy Specimen grids with the treponemes were then prepared by the multiple drop technique (3) 1 per cent ammonium molybdate pH 7 2 was used as negative stain

The specimens were coded so that the operator at the electron microscope was without knowledge of the particular treatment of the cells of each specimen Electron microscopy was carried out on a Philips EM 200 electron microscope and suitable fields of view were taken at random at primary magnifications of 9000 or 16 000 × Negatives were obtained on Kodak Fine Grain Release Positive Film Type 5302 and were as a routine 10 × enlarged photographically For this study approximately 400 electron micrographs were examined

RESULTS

In experiments carried out as described it was found that a minimum incubation time of 8 hours with unheated GPS and serum Cop 111 present in the incubation medium was required to obtain immobilization of all the treponemes (100 per cent immobilization)

Table 1 shows the contents of the different tubes including the controls In the different experiments the treponemes which were incubated in plain basal medium for 8 hours showed a percentage of motile cells varying between 60 and 100 In the electron microscope the cells showed the appearance previously described for normal undamaged cells of *T pallidum* (2) *i e* a pointed striated tip (zone 1) a front part of the cytoplasmic body with no flagella (zone 2) and an insertion region for the flagella (zone 3) In some cells however, the tip and the zone 2 part of the cytoplasmic body were slightly damaged but the majority of the cells appeared normal with the outer membrane tightly enveloping the cells No flagella were seen to pierce this membrane or to protude from the cell surface

Treponemes incubated in basal medium to which was added heated GPS or unheated GPS or serum Cop 111 (tubes 2 3 and 5, respectively in Table 1) all showed a similar normal morphology The same applied to cells incubated in basal medium to which was added either serum Np plus unheated GPS (Fig 1) or serum Cop 111 plus heated GPS (Fig 2) (tubes 4 and 6 respectively in Table 1) Dark field microscopy on cells from these tubes showed that all cells were motile (table 1)

In contrast all cells incubated in basal medium with serum Cop 111 and unheated GPS were found to be immobilized (tube 7, Table 1) By electron microscopy it was observed that these cells were swollen and the exterior of their outer membrane was covered with some fuzzy material which almost obscured the outer border (figs 3 and 4) The total

TABLE 1 *Percentage of Motile Treponemes in Different Reaction Mixtures after Various Hours of Incubation*

Tube no	1	2	3	4	5	6	7
Treponemes suspended in basal medium (ml)	0 25	0 25	0 25	0 25	0 25	0 25	0 25
Basal medium added (ml)	0 25	0 05	0 05	0	0 20	0	0
Human serum added (ml)	0	0	0	0 05 Np	0 05 Cop 111	0 05 Cop 111	0 05 Cop 111
GPS added ml	0	0 20h	0 20u	0 20u	0	0 20h	0 20u
Hours of incubation	Percentage of motile cells						
0	100	100	100	100	100	100	100
4	100	100	100	100	100	100	88
6	64	100	100	100	96	100	63
8	67	100	100	100	82	100	0
12	56	96	100	96	68	96	0

h heated GPS (56° C for 30 minutes) u unheated GPS
Np = pool of sera from healthy blood donors (see Material and Methods)
Cop 111 = serum from a syphilitic patient (7)

50 per cent of the cells were found to be swollen and their surface covered by fuzzy material All cells incubated for 8 and 12 hours were swollen and covered by the same type of fuzzy material This correlated well with the number of immobile cells determined by dark field microscopy of cells from the same samples which amounted to 0, 12, 37, 100 and 100 per cent of the cells counted (Table 1) The immunoimmobilized cells showed some indication of lysis after incubation for 12 hours (Fig 4) while those incubated for 8 hours did not All swollen cells presented similar morphological features

TABLE 2 *Effect of Dilution of Serum Cop 111 on the Motility of Treponemes after 8 Hours of Incubation*

Tube No	Serum Cop 111 diluted						
	1/1	1/2	1/4	1/8	1/16	1/32	1/64
5	100						
6	100	100	100	100	100	100	100
7	4	58	74	92	100	100	100

Each figure gives the percentage of motile cells determined by dark field microscopy on portions of each tube Contents of tubes as in Table 1

The percentage of motile treponemes after incubation for 8 hours with heated or unheated GPS and varying concentrations of serum Cop 111 is shown in Table 2 All treponemes which were motile presented an appearance typical of normal *T pallidum* cells Examination in the electron micro scope showed that the percentage of swollen cells with fuzzy material on their surface corresponded amazingly well to the percentage of immobile cells found by dark field microscopy (Table 2)

DISCUSSION

Specimens for electron microscopy were prepared as soon as possible after the 8 hours incubation period so that the morphological changes observed most probably could be attributed to the effects of the immunoimmobilization and to a much less extent to autolysis of the cells Immunoimmobilized cells from suspensions incubated for 12 hours were found to be slightly more damaged than cells incubated for 8 hours This indicates that during the 4 hours of incubation in the immobilized state, the cells were subjected to further injury from intra- or extracellular lytic agents

Cells from suspensions of the controls of the TPI test presented an ultrastructure normal for *T pallidum* cells Even cells from the control suspensions in plain basal medium which after incubation showed as many as 40 per cent immobile cells, all showed a perfectly normal morphology *Greifelt* (1) observed that treponemes incubated in basal medium to which specific antibodies and active complement were added, appeared swollen and with indistinct contours, and *Zaffiro* (13) reported alterations of the morphology of treponemes incubated with specific antibodies and complement The regular helical shape of the cells was damaged and fibrils were seen along the cells In our studies, [illegible] that a distinct gap is present between the outer membrane and the cytoplasmic body, and the surface of the outer membrane is covered by fuzzy material These characteristic changes have not been seen in other preparations of *T pallidum* cells studied by electron microscopy, irrespective of whether the examined cells were unfixed fixed with different fixatives or treated with various enzymes or detergents (2)

Figs 3–5 show cells immunoimmobilized after incubation in basal medium plus human syphilis serum and unheated GPS The cells appear swollen and the surface of their outer membrane (OM) is covered by some fuzzy material Note that the fuzzy layer gives the cell border a blurred and indistinct appearance (arrows) The three zones normally present at the ends of the treponeme are difficult to visualize in the immunoimmobilized cells

Fig 3 Treponeme after 8 hours of incubation

Fig 4 Treponeme after 12 hours of incubation This cell shows a slight tendency to lysis compared with the cell in Fig 3

Fig 5 Treponeme incubated for 8 hours The border of the outer cell membrane is seen to continue all around the [illegible]

3 1 2 3 MO F

4 OM 3 F

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It is conceivable that the fuzzy material present on the surface of the immunoimmobilized cells in some way is responsible for the widening of the periplasmic space The presence of this material probably influences the osmotic barrier capacity of the outer membrane so that an uncontrolled influx of water and ions occurs thus creating the gap between the cytoplasmic body and the outer membrane of the cell

It has been suggested that immobilization of the treponemes represents the end result of two types of sequential reactions (10) The events of the first reaction sequence lead to a sensitization of the treponemes and those of the second result in the actual immunoimmobilization (8) One or several thermolabile factors present in the unheated GPS were shown to be essential for both sensitization and immobilization (8 9) From the present experiments no morphological changes can be correlated with the sensitization process and it has also been impossible to determine whether the immunoimmobilized cells are killed or paralysed

Our experiments corroborate the previous findings that in addition to antibodies the presence of some thermolabile factor or factors from GPS is essential for the immunoimmobilization to occur (9) The morphological changes observed are considered to be specifically attributed to the immobilization process and to be caused by the combined action on the treponemal cells of antibody and at least one thermolabile factor in the GPS It is tempting to suggest that the fuzzy material covering the immunoimmobilized cell consists of antibodies present in serum from syphilitic patients which complex with factors present in the unheated GPS This hypothesis can to a certain extent be tested by immunoelectron microscopy and the results obtained with this technique will be presented in a subsequent paper

The authors wish to thank *A Knudsen* for her excellent assistance in the performance of the TPI tests We also thank *F Laursen* for excellent assistance in electron microscopy and *A G Overgaard* and *F Laursen* for expert photographic work

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Acta path microbiol scand Sect. C *87* 223–227 1979

SENDAI VIRUS HAEMOLYSIS MECHANISM OF THE INCREASED HAEMOLYSIS OBTAINED BY PRETREATMENT OF THE VIRUS WITH ANTIBODY AND COMPLEMENT

PER FADNES and GUNNAR HAUKENES

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Fadnes P & Haukenes G Sendai virus haemolysis Mechanism of the increased haemolysis obtained by pretreatment of the virus with antibody and complement Acta path microbiol scand Sect C *87* 223–227 1979

Sendai virus is haemolytic against erythrocytes from various species Pretreatment of the virus with antibody and complement (C) resulted in a sixfold increase in the haemolysis The viral haemolysin (HL) could be inactivated by heating the virus at 37° C for 5 h HL-inactivated virus became haemolytic again when pre treated with antibody and C This acquired haemolytic capacity corresponded to the enhanced haemolysis shown by intact virus It is concluded that the enhanced haemolytic activity is due to a C mediated indirect haemolysis and is not dependent on the fusion process The possibility of cell damage *in vivo* from virus antibody-C complexes is discussed

Key words Sendai virus haemolysis immune complex

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Sendai virus produces a factor which causes fusion of animal cells and of virus and erythrocytes called the fusion factor or haemolysin (HL) respectively Prior to haemolysis the virus membrane fuses with the erythrocyte membrane The normal haemolytic activity of Sendai virus is thus ascribed to lesions in that part of the virus membrane which has become inserted into the erythrocyte membrane (1 10) *Homma et al* (6) have reported that virus harvested at an early stage had no lesions and lacked the activity to lyse erythrocytes After freezing and thawing or when harvested later the virus became haemolytic This also occurred when virus was pre treated with antibody and complement (C) This led the authors to conclude that C mediated lesions of the virus membrane might cause leakage of haemoglobin (2 10)

In the present study we have examined the mechanisms involved in the enhancement of Sendai virus haemolysis caused by antibody and C

MATERIALS AND METHODS

Virus

Parainfluenza I virus (Sendai) was propagated in the chick allantoic cavity for 2 to 3 days In some experiments the virus was purified by pelleting in the ultracentrifuge at 100 000 × g for 1 h The virus was quantified by haemagglutination (HA) using chick erythrocytes

Antisera

Antiserum against Sendai virus was raised in rabbits by intravenous injection of virus-containing allantoic fluid The antiserum gave HA inhibition (HI) titres of 512 to 1024 against 4 HA units of Sendai virus [illegible]

Complement (C)

Fresh guinea pig serum or guinea pig serum stored at −70° C was used as source of complement

Buffer

Studies of C activity were performed using a barbital buffer of pH 7 3 made isotonic by 0 8 per cent NaCl and supplemented with 0 15 mM $CaCl_2$ and 0 5 mM $MgCl_2$ (C-buffer) To stop the activation of C, 10 mM of ethylenediaminetetraacetic acid (EDTA) was added

Sendai Virus Haemolysis

Pre-treatment of Sendai virus by antiserum and C was performed in glass tubes in a total reaction volume of 0 3 ml, consisting of 0 1 ml Sendai virus in allantoic fluid (HA titre 1024) 0 1 ml diluted antiserum and 0 1 ml diluted C Controls in which antiserum or C was replaced by C-buffer were always included

The reaction mixture was incubated at 37° C for 1 h Thereafter 2 ml of a 2 per cent suspension of chick erythrocytes in the barbital buffer with 10 mM EDTA and without Ca^{++} and Mg^{++} was added The tubes were incubated for a further hour at 37° C and shaken repeatedly to prevent sedimentation of the erythrocytes The erythrocytes were then pelleted at 3 600 × *g* for 5 min, and the optical density (OD) of the supernatant was read in a Hitachi spectrophotometer at 540 nm

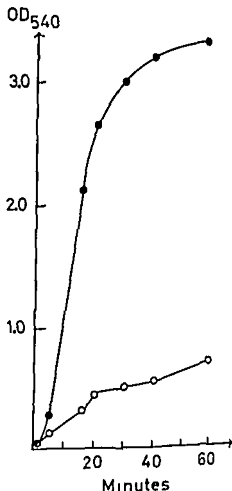


Fig 1 Haemolytic activity (OD_{540}) of Sendai virus before and after treatment with antibody and C O—O Untreated virus ●—● virus treated with antiserum to influenza virus B/Lee (1 16) and C (1 16) for 1 h Haemolysis was recorded at intervals during a 60 min incubation period

EXPERIMENTS AND RESULTS

Haemolytic Activity of Untreated and Antibody-C Treated Virus

The reaction between virus and erythrocytes was stopped at different times (2 to 60 min) and the haemolysis recorded at OD_{540} A representative experiment is shown in Fig 1

The haemolytic activity of antibody-C-treated virus was low during the first 5 min, followed by a rapid and linear increase between 5 and 20 min Further incubation up to 60 min gave only a slight increase Pre-treatment of virus with antibody and C resulted in an approximately sixfold increase in haemolysis

Haemolytic Activity after Removal of Soluble Serum Factors

The possibility exists that C3 and C5 convertases already present on the virus surface might split C3 and C5 in the reaction mixture after contact between virus and erythrocytes In this way the haemolytic C5–9 complex might be built up on the erythrocyte membrane To examine this possibility

haemolytic activity (Fig 2)

Haemolytic Activity of HA- and HL-Negative Virus

Preliminary experiments indicated that the HL was very sensitive to heat At 56° C the HL activity was lost almost immediately, while incubation at 37° C led to a gradual loss of activity in the course of 5 to 7 h At 56° C the HA activity was also lost, whereas it was still present after 24 h at 37° C

It has been reported that the treatment of measles virus with 4 7 per cent formaldehyde for 30 min at 37° C completely inactivates the HL, while some HA activity is retained (8) Sendai virus appeared to be more sensitive to formaldehyde since a 0 25 per cent solution inactivated both HL and HA

Since the HA activity was retained after incubation at 37° C, this procedure was used to study the effect of HL inactivation Treatment at 56° C or by

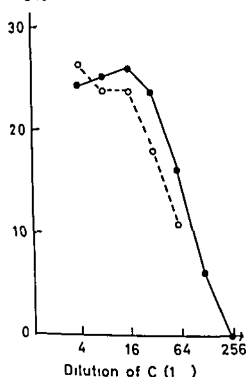


Fig 2 Increase in haemolytic activity (OD_{540}) by treating Sendai virus with antibody (anti influenza virus B/Lee 1 16) and C Haemolysis was recorded before and after removal of soluble serum factors by pelleting the virus at 100 000 × *g* for 1 h ●—● Virus treated with antibody and C O--O virus treated as above after the removal of soluble serum factors

formaldehyde was performed in order to inactivate both HA and HL

Untreated virus and virus treated with antibody and C were incubated at 37° C for different times before the haemolysis was measured (Fig 3) After 5 h at 37° C untreated virus had lost its haemolytic activity (HL negative virus) Antibody and C- [illegible]

[illegible] (Fig 4) The difference in haemolytic activity between these two virus preparations corresponded well with that obtained when using intact virus not treated with antibody and C Treatment with antiserum to Sendai virus resulted in a greater difference between HL negative and intact virus than treatment with antiserum to the host antigen (Fig 4)

HA- and HL inactivated virus (56° C or formaldehyde treatment) showed no haemolytic activity even after treatment with antibody and C The same effect was observed when high concentrations of antiserum to Sendai virus and intact virus were used possibly due to inhibition of the contact between virus and erythrocytes

Physical Treatment of the Virus

In order to examine whether increased disruption of the virus had any influence on its haemolytic activity different physical treatments of the virus were performed

Treatment with ultrasonics (22 kc) for 2 to 120 s or freezing and thawing several times (1 to 10) had no influence on the haemolytic activity of the virus

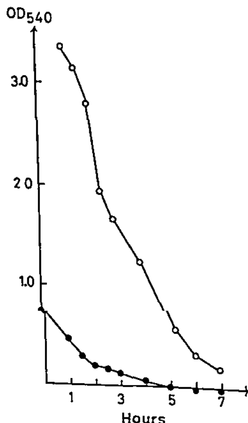


Fig 3 Haemolytic activity (OD_{540}) of untreated and antibody-C treated Sendai virus after different times of incubation at 37° C ●—● Untreated virus O—O virus treated with antiserum to influenza virus strain B/Lee (1 16) and C (1 16)

Complement (C)

Fresh guinea pig serum or guinea pig serum stored at −70° C was used as source of complement

Buffer

Studies of C activity were performed using a barbital buffer of pH 7 3 made isotonic by 0 8 per cent NaCl and supplemented with 0 15 mM $CaCl_2$ and 0 5 mM $MgCl_2$ (C buffer) To stop the activation of C 10 mM of ethylenediaminetetraacetic acid (EDTA) was added

Sendai Virus Haemolysis

Pre treatment of Sendai virus by antiserum and C was performed in glass tubes in a total reaction volume of 0 3 ml consisting of 0 1 ml Sendai virus in allantoic fluid (HA titre 1024) 0 1 ml diluted antiserum and 0 1 ml diluted C Controls in which antiserum or C was replaced by C buffer were always included

The reaction mixture was incubated at 37° C for 1 h Thereafter 2 ml of a 2 per cent suspension of chick erythrocytes in the barbital buffer with 10 mM EDTA and without Ca^{++} and Mg^{++} was added The tubes were incubated for a further hour at 37° C and shaken repeatedly to prevent sedimentation of the erythrocytes The erythrocytes were then pelleted at 3 600 × *g* for 5 min and the optical density (OD) of the supernatant was read in a Hitachi spectrophotometer at 540 nm

EXPERIMENTS AND RESULTS

Haemolytic Activity of Untreated and Antibody C Treated Virus

The reaction between virus and erythrocytes was stopped at different times (2 to 60 min) and the haemolysis recorded at OD_{540} A representative experiment is shown in Fig 1

The haemolytic activity of antibody C treated virus was low during the first 5 min followed by a rapid and linear increase between 5 and 20 min Further incubation up to 60 min gave only a slight increase Pre treatment of virus with antibody and C resulted in an approximately sixfold increase in haemolysis

Haemolytic Activity after Removal of Soluble Serum Factors

The possibility exists that C3 and C5 convertases already present on the virus surface might split C3 and C5 in the reaction mixture after contact between virus and erythrocytes In this way the haemolytic C5–9 complex might be built up on the

... To examine this possibility ... xture were ... ated virus ... at 100 000 × *g* for 1 h followed by ... ing and re pelleting No influence was observed on the haemolytic activity (Fig 2)

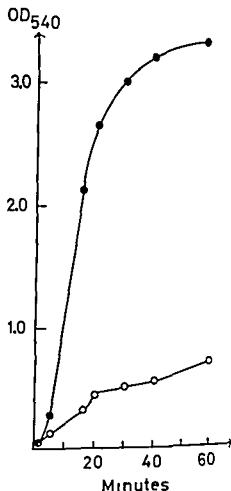


Fig 1 Haemolytic activity (OD_{540}) of Sendai virus before and after treatment with antibody and C O—O Untreated virus ●—● virus treated with antiserum to influenza virus B/Lee (1 16) and C (1 16) for 1 h Haemolysis was recorded at intervals during a 60 min incubation period

Haemolytic Activity of HA and HL Negative Virus

Preliminary experiments indicated that the HL was very sensitive to heat At 56° C the HL activity was lost almost immediately while incubation at 37° C led to a gradual loss of activity in the course of 5 to 7 h At 56° C the HA activity was also lost whereas it was still present after 24 h at 37° C

It has been reported that the treatment of measles virus with 4 7 per cent formaldehyde for 30 min at 37° C completely inactivates the HL while some HA activity is retained (8) Sendai virus appeared to be more sensitive to formaldehyde since a 0 25 per cent solution inactivated both HL and HA

Since the HA activity was retained after incubation at 37° C this procedure was used to study the effect of HL inactivation Treatment at 56° C or by

has been shown to occur with antibody and C treated *Salmonella typhimurium* and erythrocytes (9) It has been suggested that the haemolysis was due to free C5–9 or to C5–9 bound to bacteria However *Kolb & Muller Eberhard* (7) have shown that free C5–9 is not haemolytic against erythrocytes We think that the above mentioned results with *S typhimurium* may be explained by the binding of C5–9 by small wall fragments not pelleted in the centrifuge

The indirect haemolysis observed by antibody and C treated Sendai virus raises the possibility of cell lysis by the same mechanism *in vivo* Virus antibody complexes which have activated C and are still able to attach to susceptible cells may cause lysis of these cells if the complexes are not phagocytised If this mechanism is operative *in vivo* damage of non infected tissue from circulating virus antibody C complexes may result.

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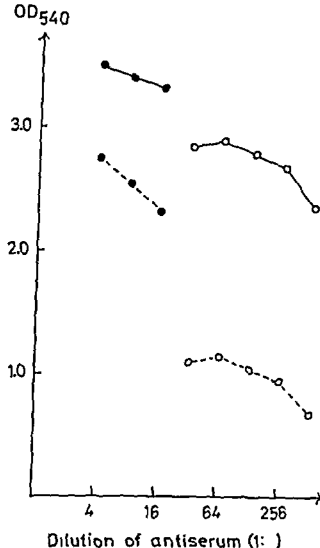


Fig 4 Comparison of haemolytic activity (OD_{540}) of intact Sendai virus and HL negative virus (treated for 24 h at 37° C) after treatment with antibody and C ●—● Intact virus treated with antiserum to influenza B/Lee and C (1 16) ●---● HL negative virus treated as above, O—O intact virus treated with antiserum to Sendai virus and C (1 16) O---O HL negative virus treated ad above

DISCUSSION

The C-induced haemolysis cannot have been caused by heterophil antibody to erythrocyte antigens since this reaction does not take place in the presence of EDTA The C must therefore have been activated by the reaction of virus with its antibody

If the enhanced haemolysis observed by treating Sendai virus with antibody and C was due to an increased number of lesions in the virus membrane as has been previously reported (10) it could be expected that physical disruption of the membrane would have the same effect In our studies neither treatment with ultrasonics nor repeated freezing and thawing had any influence on the haemolytic activity of the virus

All the haemolytic activity of the virus was inactivated after incubation for several hours at 37° C (HL-negative virus), but treatment of this virus with antibody and C rendered it haemolytic again Thus this C-mediated haemolysis cannot be due to an increased number of lesions in the virus membrane It has been shown previously that antibodies to the host antigen give rise to holes in the Sendai virus membrane in the presence of C (5) Similar holes were produced using antiserum to Sendai virus (unpublished)

The difference obtained by inactivating HL before and after treatment with antibody and C further supports the view that the C-induced haemolysis does not depend on a fusion process Antibody- and C-treated virus lost almost all its haemolytic activity after 7 h at 37° C, whereas HL-negative (24 h at 37° C) virus became strongly haemolytic when treated with antibody and C Thus this difference cannot be explained by inactivation of HL, but may be due to the inactivation of activated C complexes (C5–9) after several hours at 37° C

The haemolysis by HL-negative virus was weaker than that obtained with intact virus when C and antiserum to the host antigen were used The difference in haemolytic activity was similar to the haemolytic activity obtained with intact untreated virus This may indicate that the C-induced haemolysis is the same regardless of whether the HL is intact or inactivated Using antiserum to whole Sendai virus the reduction of C-induced haemolysis with HL-negative virus was greater than that obtained by using antiserum to the host antigen This lowered haemolysis may be explained by an inactivation of the antigenic part of the HL It has been shown with measles virus that HL-antigen-antibody activates the classical C pathway (3)

Removal of soluble serum factors from the reaction mixture by pelleting the virus had no effect on the haemolytic activity This indicates that C-mediated haemolysis cannot be due to active C3 or C5 convertases on the virus membrane splitting *C3* and C5 and thus forming the membrane complex C5–9 on the erythrocyte

The only explanation of the increase in haemolysis after treatment of the virus with antibody and C seems to be indirect haemolysis due to active *C5–9* complexes on the virus membrane *The C5–9* complex may act on the erythrocyte membrane from the attached virus or it may move from the virus to the erythrocyte in an equilibrium reaction Both theories would require a close contact between

has been shown to occur with antibody and C-treated *Salmonella typhimurium* and erythrocytes (9) It has been suggested that the haemolysis was due to free C5–9 or to C5–9 bound to bacteria However *Kolb & Muller Eberhard* (7) have shown that free C5–9 is not haemolytic against erythrocytes We think that the above mentioned results with *S typh murium* may be explained by the binding of C5-9 by small wall fragments not pelleted in the centrifuge

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Acta path microbiol scand Sect C 87 229–233 1979

IgA AND IgG ANTIBODIES AGAINST SURFACE ANTIGENS OF *PSEUDOMONAS AERUGINOSA* IN SPUTUM AND SERUM FROM PATIENTS WITH CYSTIC FIBROSIS

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Schiøtz P O Høiby N Permin H & Wiik A IgA and IgG antibodies against surface antigens of *Pseudomonas aeruginosa* in sputum and serum from patients with cystic fibrosis Acta path microbiol scand Sect C 87 229–233 1979

Eleven cystic fibrosis (CF) patients chronically infected in the lungs with mucoid *Pseudomonas aeruginosa* and presenting multiple precipitins in serum against this bacterium (CF + P) and 10 CF patients without *P aeruginosa* infection (CF–P) had their serum and sputum sol phase specimens

($p < 0.01$) The titre of IgA antibodies in the sputum was higher than in serum in 3 cases indicating local pulmonary production of specific IgA antibodies The role of the demonstrated antibodies in the local pulmonary immune defense mechanisms and the possible patogenesis of the pulmonary tissue damage in CF patients is discussed

Key words Cystic fibrosis *Pseudomonas aeruginosa* antibodies sputum

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Chronic pulmonary infection with mucoid *Pseudomonas aeruginosa* is known to occur in most cystic fibrosis (CF) patients sooner or later and is associated with a poor prognosis (16) In spite of a pronounced humoral immune response reflected by high levels of antibodies against *P aeruginosa* in

keeping the infection localized to the respiratory tract This suggests that an immunological defect might exist locally in the lung

Using crossed immunoelectrophoresis with inter ... found precipitating antibodies against water soluble antigens of *P aeruginosa* in the sputum sol phase of CF patients with chronic *P aeruginosa* lung infection (20) However it was not settled whether these antibodies were directed against surface antigens of *P aeruginosa* and the immunoglobulin class was not investigated

In the present work we therefore wanted to investigate the occurrence of IgA and IgG class of antibodies against surface antigens of the patients own *P aeruginosa* strain in both serum and sputum sol phase specimens The results were compared with those of a control group of CF patients without *P aeruginosa* infection

TABLE 1 *Titres of IgA Antibodies against Surface Antigens of* Pseudomonas aeruginosa *in Serum and Sputum Sol Phase and Number of* P aeruginosa *Precipitins in Serum from 21 Cystic Fibrosis Patients with (CF + P) and without (CF–P)* P aeruginosa *Lung Infection*

Patient no	No of *P aeruginosa* precipitins in serum	Serum titre[a])	Sputum sol phase titre[a])
CF–P			
1	1	4	0
2	0	2	0
3	1	32	4
4	0	1	1
5	0	8	0
6	0	32	2
7	0	2	0
8	0	4	0
9	0	0	0
10	1	0	0
CF + P			
11	20	256	8
12	24	128	64
13	39	512	64
14	16	512	8
15	20	4096	128
16	48	64	16
17	31	64	256
18	17	256	0
19	24	128	8
20	41	64	512
21	30	256	512

[a]) 0 means negative while the positive titres are indicated as 1 (positive undiluted) or with the reciprocal value of the highest positive dilution

TABLE 2 *Titres of IgG Antibodies against Surface Antigens of* Pseudomonas aeruginosa *in Serum and Sputum Sol Phase and Number of* P aeruginosa *Precipitins in Serum from 21 Cystic Fibrosis Patients with (CF + P) and without (CF–P)* P aeruginosa *Lung Infection*

Patient no	No of *P aeruginosa* precipitins in serum	Serum titre[a])	Sputum sol phase titre[a])
CF–P			
1	1	32	4
2	0	2	0
3	1	0	0
4	0	4	0
5	0	2	0
6	0	128	1
7	0	0	0
8	0	4	0
9	0	1	0
10	1	0	0
CF + P			
11	20	1024	32
12	24	2048	16
13	39	4096	32
14	16	2048	8
15	20	256	16
16	48	2048	16
17	31	1024	32
18	17	512	4
19	24	2048	16
20	41	1024	64
21	30	256	16

[a]) symbols as in Table 1

against surface antigens of the patients own *P aeruginosa* strain in serum and sputum of CF + P patients The presence of low titered *P aeruginosa* antibodies in CF–P patients is well in agreement with our knowledge of cross reacting antibodies (15)

The titres of IgA and IgG antibodies against surface antigens of *P aeruginosa* in the sera from CF + P patients were significantly higher than the titres in the corresponding sputa. This may indicate that the presence of IgA and IgG antibodies in the sputum sol phase is at least to some degree a result of exudation from serum through an inflamed mucous membrane However three patients showed higher titres of IgA antibodies in their sputum sol phase than in serum In these cases some of the IgA class antibodies are probably produced locally by the bronchus associated lymphoid tissue. The presence of *P aeruginosa* antigens in the sputum and the possibility of immune complex formation between *P aeruginosa* antigens and specific IgA anti *P aeruginosa* antibodies may lead to an underestimation of the locally produced specific IgA

The number of *P aeruginosa* precipitins in serum from CF + P patients only showed correlation to the IgG titres in sputum – and the rather low R value (0 65) may reflect the differences in the 2 methods employed to detect these antibodies

One of the main roles of IgA antibodies is to inhibit bacterial adherence to the epithelial surfaces In the second line of defense against *P aeruginosa* infections the antibodies opsonize the bacteria and thus promote phagocytosis of the bacteria by

PATIENTS AND METHODS

Patients

Twenty-one CF patients were included in the study All patients had a typical history of CF and markedly elevated sweat electrolytes in repeated tests (9)

(i) One group consisted of 11 CF patients (8 males and 3 females, median age 13 years, range 10–26 years) suffering from chronic pulmonary infection with mucoid strains of *P aeruginosa* (CF + P) and exhibiting more than 10 different precipitins in serum against water-soluble antigens from these bacteria The patients have been followed as previously described (12, 13) Mean duration of the *P aeruginosa* respiratory tract infection was 4 5 years, range 1–8 years

(ii) The other group consisted of 10 CF patients (5 males, 5 females, median age 14 years, range 10–21) without *P aeruginosa* lung infection (CF–P) and without serum precipitins against *P aeruginosa* antigens In 6 of the CF–P patients *Staphylococcus aureus Haemophilus influenzae* or *Klebsiella pneumoniae* were isolated from the respiratory tract during this study

Sputum

A 3-h-sample of sputum was collected at 4°C from each patient between 8 a m and 11 a m as described previously (6, 20) Each sample was subjected to bacteriological examination and the origin of the specimens from the lower respiratory tract was confirmed by studying the epithelial cells present (13, 20) Sol phase of sputum was obtained by ultracentrifugation at 120 000 × *g* at 4°C for 4 h and stored in small aliqouts at – 80°C (19)

Crossed Immunoelectrophoresis

The occurrence of circulating precipitating antibodies in serum against *P aeruginosa* were investigated by means of crossed immunoelectrophoresis (microtechnique) as described previously (14)

Immunofluorescence Technique

Eleven strains of *P aeruginosa* from the 11 CF + P patients were cultured on solid media (Truche) (12) at 35°C overnight Each strain was suspended in sterile 0 154 M NaCl (approximately 10^9 CFU/ml) and kept at –30°C until used for the experiments Subcultures on solid media from the suspensions showed that most of the cells at that time had reverted to the non-mucoid dissociants, although they were mucoid at the time of isolation from the patients

After thawing, the organisms were washed 2 times in phosphate-buffered saline (PBS) with 1% human serum albumin, pH 7 4 A suspension was prepared containing approximately 10^6 CFU/ml 100 µl of this bacterial suspension was incubated with 100 µl of serum or sputum sol phase at 37°C for 30 minutes followed by two washings in PBS for 10 minutes at 1000 *g* 50 µl of conjugate (see below) was added and the tubes were again incubated for 30 minutes at 4°C, followed by a second set of washings The organisms were applied on glass slides, air-dried and mounted with a mixture of glycerol and PBS (1 2), under cover slips The samples were studied for presence of IgA and IgG antibodies to *P aeruginosa* using fluorescein isothiocyanate labelled IgG fractions of rabbit antisera specific for human α and γ chains from Dakopatts, Denmark The specificity of the conjugates had been checked by crossed immunoelectrophoresis against normal human serum and direct immunofluorescence technique on monoclonal bone marrow specimens from patients with myelomas (IgG, IgA) (24) Results were read in a Leitz Orthoplan microscope equipped for incident light illumination as earlier described (21)

In patients chronically infected with mucoid strains of *P aeruginosa* the autologous strain was used as antigen source Ten of these *P aeruginosa* strains were used randomly as antigens in the tests with sputum and serum from CF–P patients

All sera were examined undiluted and diluted 1 16, and sol phases undiluted and diluted 1 2, 1 4 and 1 8 Twofold titration of positive samples was performed using PBS as diluent

Statistical Methods

The Mann-Whitney test, Wilcoxon's test for pair differences and Spearman's correlation coefficient R were employed (7) Level of significance 5 per cent (double tailed test)

RESULTS

The titres of antibodies of the IgA class as well as of the IgG class against surface antigens of *P aeruginosa* were significantly higher in serum and sputum of the CF + P patients than in serum and sputum of the CF–P patients ($p<0\ 01$) (Table 1 & 2)

The CF + P patients furthermore had significantly higher titres of IgA antibodies ($p<0\ 05$) and of IgG antibodies ($p<0\ 01$) against surface antigens of *P aeruginosa* in serum than in sputum (Table 1 & 2) A correlation between the titre in serum and the corresponding titre in sputum of the CF + P patients could not be demonstrated neither with regard to IgA nor with regard to IgG antibodies against surface antigens of *P aeruginosa*

The number of *P aeruginosa* precipitins in serum of the CF + P patients (Table 1 & 2) was compared with the titre of IgA and IgG antibodies in serum as well as in sputum A positive correlation was found only between the IgG titres in sputum and the number of *P aeruginosa* precipitins in serum ($R = 0\ 65$, $p<0\ 05$)

DISCUSSION

The present study demonstrates in accordance with the results of *Harn et al* (11) the presence of high titres of antibodie

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1	1	4	0
2	0	2	0
3	1	32	4
4	0	1	1
5	0	8	0
6	0	32	2
7	0	2	0
8	0	4	0
9	0	0	0
10	1	0	0
CF+P			
11	20	256	8
12	24	128	64
13	39	512	64
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1	1	32	4
2	0	2	0
3	1	0	0
4	0	4	0
5	0	2	0
6	0	128	1
7	0	0	0
8	0	4	0
9	0	1	0
10	1	0	0
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[illegible] the organisms were washed 2 times in [illegible] 1% human serum [illegible] prepared containing approximately 10^6 CFU/ml [illegible] µl of this bacterial suspension was incubated with 100 µl of serum or sputum sol phase at 37°C for 30 minutes followed by two washings in PBS for 10 minutes at *1000 g*. *50 µl* of [illegible] were again [illegible] nd [illegible] ss [illegible] of slides, air-dried and mounted with [illegible] and PBS (1:2), under cover slips. The samples were studied for presence of IgA and IgG antibodies to *P. aeruginosa* using fluorescein isothiocyanate labelled IgG fractions of rabbit antisera specific for human α and γ chains from Dakopatts, Denmark. The specificity of the conjugates had been checked by crossed immunoelectrophoresis against normal human serum and direct immunofluorescence technique on monoclonal bone marrow specimens from patients with myelomas (IgG, IgA) (24). Results were read in a Leitz Orthoplan microscope equipped for incident light illumination as earlier described (21).

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DISCUSSION

The present study demonstrates in accordance with the results of *Hann et al.* (11) the presence of high titres of antibodies of both the IgA and IgG classes

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6	0	32	2
7	0	2	0
8	0	4	0
9	0	0	0
10	1	0	0
CF + P			
11	20	256	8
12	24	128	64
13	39	512	64
14	16	512	8
15	20	4096	128
16	48	64	16
17	31	64	256
18	17	256	0
19	24	128	8
20	41	64	512
21	30	256	512

[a]) 0 means negative while the positive titres are indicated as 1 (positive undiluted) or with the reciprocal value of the highest positive dilution

TABLE 2 *Titres of IgG Antibodies against Surface Antigens of* Pseudomonas aeruginosa *in Serum and Sputum Sol Phase and Number of* P aeruginosa *Precipitins in Serum from 21 Cystic Fibrosis Patients with (CF + P) and without (CF–P)* P aeruginosa *Lung Infection*

Patient no	No of *P aeruginosa* precipitins in serum	Serum titre[a])	Sputum sol phase titre[a])
CF–P			
1	1	32	4
2	0	2	0
3	1	0	0
4	0	4	0
5	0	2	0
6	0	128	1
7	0	0	0
8	0	4	0
9	0	1	0
10	1	0	0
CF + P			
11	20	1024	32
12	24	2048	16
13	39	4096	32
14	16	2048	8
15	20	256	16
16	48	2048	16
17	31	1024	32
18	17	512	4
19	24	2048	16
20	41	1024	64
21	30	256	16

[a]) symbols as in Table 1

against surface antigens of the patients own *P aeruginosa* strain in serum and sputum of CF + P patients The presence of low titered *P aeruginosa* antibodies in CF–P patients is well in agreement with our knowledge of cross reacting antibodies (15)

The titres of IgA and IgG antibodies against surface antigens of *P aeruginosa* in the sera from CF + P patients were significantly higher than the [illegible]

by the bronchus associated lymphoid tissue The presence of *P aeruginosa* antigens in the sputum and the possibility of immune complex formation between *P aeruginosa* antigens and specific IgA anti *P aeruginosa* antibodies may lead to an underestimation of the locally produced specific IgA

The number of *P aeruginosa* precipitins in serum from CF + P patients only showed correlation to the IgG titres in sputum – and the rather low R value (0 65) may reflect the differences in the 2 methods employed to detect these antibodies

One of the main roles of IgA antibodies is to inhibit bacterial adherence to the epithelial surfaces In the second line of defense against *P aeruginosa* infections the antibodies opsonize the bacteria and thus promote phagocytosis of the bacteria by

neutrophils or macrophages (3, 4, 25) Another protective role of antibodies is to participate in the complement dependent bactericidal activity of serum against *P aeruginosa* (17) Sera from CF + P patients seem to be able to opsonize *P aeruginosa* and thus promote phagocytosis of the bacteria by human neutrophils (18), whereas CF sera possibly are defective as a source of opsonins when rabbit alveolar macrophages are mixed with *P aeruginosa* cells (2, 5) Similarly, sera from most CF patients are bactericidal against the patients own *P aeruginosa* strain However, in a few cases »bactericidal blocking« antibodies seem to occur in CF patients sera which are less efficient than normal sera in killing the autologous strain (17)

It should be noted that although all the *P aeruginosa* strains primarily were mucoid the mucoid substance is not firmly bound to the bacterial cells, but diffuses into the environment particularly in a fluid phase system (8) In the present study we have therefore probably worked mainly with non mucoid strains The dissociation of *P aeruginosa* from mucoid to non mucoid variants is also known to occur in vivo and may play an important role in the host parasite relationship (1, 8, 10, 13, 14, 17) The present results, therefore do not tell whether antibodies against mucoid substance are present in serum and sputum of CF patients Further studies should be undertaken to elucidate this problem

The presence of antibodies in sputum from CF + P patients against surface antigens of *P aeruginosa* supports the concept that immune complex formation between *P aeruginosa* antigens and antibodies may take place in the lungs in these patients even if the antigens are situated on the surface of whole bacterial cells according to the present results Immune complex activity in serum and sputum from CF + P patients have previously been demonstrated (21, 22) Furthermore complement split products have been found in sputum from CF + P patients (23) possibly due to complement activation by immune complexes consisting of *P aeruginosa* antigens and *P aeruginosa* antibodies Deposits of immune complexes have also been demonstrated in the lungs and trachea from CF patients (19)

In conclusion this study shows that insufficient immune elimination of *P aeruginosa* from the respiratory tract in CF + P patients does not seem to be due to lack of antibodies against surface antigens of *P aeruginosa* Future studies should be designed to examine the functional capacity of these antibodies to support phagocytosis to initiate complement dependent bacteriolysis or to inhibit the attachment of *P aeruginosa* to mucosal surfaces

This work was supported by *The Danish Medical Research Council* (project no 512 5267 6623 and 8115) *The National Association for the Fight against Tuberculosis The Paul Bergsøe Foundation The National Danish Association against Cystic Fibrosis* and *The National Danish Association against the Rheumatic Diseases*

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EFFECT OF PREGNANCY ZONE PROTEIN ON LEUCOCYTE MIGRATION INHIBITION, LYMPHOCYTE TRANSFORMATION AND ROSETTE FORMATION BY LYMPHOCYTES

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Birkeland S A Teisner B Schilling W Kemp E Pedersen G Thomsen & Svehag S-E Effect of pregnancy zone protein on leucocyte migration inhibition lymphocyte transformation and rosette formation by lymphocytes Acta path microbiol scand Sect. C 87 235–240 1979

Pregnancy zone protein from pregnancy serum and control preparations from male serum were tested in lymphocyte transformation tests in a system for rosette formation by T and B lymphocytes and in leucocyte migration inhibition tests using PPD induced inhibition PZ-protein preparations caused a dose dependent inhibition of lymphocyte transformation after stimulation with PHA PPD and MLC Inh bit on was mo

... ations from male serum

Key words Pregnancy zone protein leucocyte migration inhibition lymphocyte transformation E and EAC rosette formation

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There is an increasing interest in the study of immunological mechanisms allowing the mother to prevent rejection of the histoincompatible fetus (1 8) This effect has been attributed to a number of different factors including a modified cellular immune response and production of several subs tances with immunosuppressive properties

Among the substances with potential immuno-suppressive properties that in pregnancy are ...

associated relatively high molecular weight glyco-protein of α_2-mobility (14) The serum concentration of this protein increases markedly during pregnancy and reaches a maximum in the 38th week of pregnancy (12 15)

In the present investigation the immunosuppres sive potentials of the protein was studied in in vitro models using lymphocyte transformation rosette formation tests for T and B lymphocytes and leucocyte migration inhibition tests

MATERIALS AND METHODS

Materials

The protein fractions used were obtained from venous blood of 30 healthy pregnant and 20 non pregnant women and from 20 normal men

$NaHCO_3$ The agarose layers (5 mm thickness) were incubated at 37° C for 1½ hours in 2% CO_2 in air Wells (2 3 mm) were punched in the gel and 7 µl aliquots of both antigen incubated suspensions and control suspensions containing 1 5 × 10^6 leucocytes were added The gels were incubated at 37° C in 2% CO_2 and 95% humidity for 18 hours The area of migration was projected onto paper of uniform quality and measured by cutting and weighing the paper image Migration indices (MI) were calculated from quadruplicate tests as ratios of m gration areas in antigen-containing cultures to migra tion areas in control cultures without antigen

Incubation of Leucocytes with PZ protein Preparations

Leucocytes were incubated with protein preparations for 30 min at 37° C before during or after PPD stimulation and without subsequent washing The final PZ concentration during testing was 10% of the concentration given for the preparations due to dilution with cell suspensions and antigen

Stat st cs

Each type of experiment was carried out as 2–8 individual determinations The results are given as means ± 1 SD Comparisons of group results were made using the T test of pairs and the Mann Whitney non parametric test.

RESULTS

Inh bitory Effect of PZ protein Preparations on Lymphocyte Transformation

A PZ protein concentration of 1 0 mg/ml was used Table 1 shows the results of one of the two experiments performed both giving similar results Significant inhibition by the control preparation from male serum (M S_2) was seen in all three forms of

ι om tests using untreated lymphocytes ($p<0\ 01$) and from tests using the male serum fraction ($p<0\ 01$)

• Variation of incubation time with comparison between 3 hours pre incubation with PZ protein preparations before stimulation and addition of PZ protein simultaneously with stimulation did not produce significantly differing results Nor did washing the cells after pre incubation

Dose response Effect of PZ protein Preparations in Lymphocyte Transformation Tests

PZ protein was used in serial 2 fold dilutions starting with a concentration of 0 75 mg PZ protein/ml and the experiments were performed with three different lymphocyte populations The results after stimulation with PHA PPD and in MLC are shown in Fig 1 In all three cases a dose dependent inhibition was observed but the inhibi tion in the PHA experiments was only partial

Rosette Formation by Lymphocytes Incubated with PZ protein Preparations and Control Preparations

Four experiments were performed with a PZ protein concentration of 1 5 mg/ml The lymphocy tes were incubated with this preparation either for 90 minutes before testing or simultaneously with testing All experiments showed a tendency towards a shift in rosette formation by T lymphocytes in the direction of fewer rosettes with ≥ 3 erythrocytes after treatment (Table 2) This shift was significant ($p<0\ 05$) when comparing lymphocytes preincuba ted with PZ protein with untreated lymphocytes The number of null cells was significantly increased both for tests using PZ protein and for tests using control preparations

Incubation with PZ protein or control prepara tions did not produce significant changes in the B rosette counts found using untreated lymphocytes

Effect of PZ protein Preparations and Control Preparations on Leucocyte Migration Inhibition

Eight experiments were performed with PZ protein preparations (2 5 mg PZ protein/ml) and 4

TABLE 1 *Effect of PZ protein Preparation from Pregnancy Serum (PS_2) and Control Preparation from Male Serum (MS_2) in Lymphocyte Transformation Tests*

Stimulation	PHA	PPD	MLC
Normal Test	18 936 ± 827	6 732 ± 1 333	8 734 ± 4 172
MS_2	7 408 ± 147	259 ± 70	3 035 ± 199
PS_2	87 ± 9	32 ± 3	94 ± 10

Lymphocytes were pre incubated with PZ ... 3 hours Normal test, test performed ... given in dpm (means ± 1 SD) of tr...

PZ-protein Preparations

The starting material was serum from pregnant women in the 35th to 38th week of gestation The PZ-protein was precipitated with 0 4% ethacridine lactate, the pH was adjusted to 6 and the sample allowed to stand at room temperature for 30 min Following centrifugation (2500 *g*) for 15 min, the precipitate was resuspended in 0 85 molar NaCl solution After re-centrifugation the dissolved precipitate was dialysed and fractionated by gel chromatography on Ultrogel ACA 22 PZ-protein containing fractions identified by fused rocket immunoelectrophoreses, were pooled and concentrated on Amicon 202, dialysed against Hepes buffer and sterile filtered The purification of PZ-protein was monitored with a quantitative electro-immunoassay using monospecific rabbit anti human-PZ-protein serum prepared and used as earlier described (10) PZ-protein made up about 60% of the total protein in the final preparations Four different PZ-protein preparations were prepared, two of them were used in all lymphocyte transformation tests and two others in studies of rosette formation and leucocyte migration inhibition Fractions from normal human male serum, prepared using the same procedures as for PZ-protein, were used as control material The concentration of PZ-protein in the control material was less than 0 1% of the total protein Total protein concentrations in both PZ-preparations and control preparations were equal in all experiments performed

Lymphocyte and Leucocyte Preparations

Lymphocytes for rosette formation were obtained from blood samples from healthy men and women Heparinised blood was mixed with an equal volume of medium TC-199 containing Hepes buffer (Flow Lab) heparin (50 I U /ml) and penicillin streptomycin (100 I U and 100 µg/ml) Iron carbonyl powder (0 1 g/ml) was added, the sample was rotated slowly for 20 min at 37° C and active phagocytes were removed with a magnet. Lymphocytes were isolated on Ficoll-Isopaque and washed twice with medium TC-199 with the additives described above plus 20% human AB serum screened for leucocyte antibodies Cell suspensions were adjusted to 3 × 10^6 lymphocytes per ml frozen and stored as described previously (3, 4)

Leucocytes for leucocyte migration tests were taken from healthy mantoux-positive men and women

Lymphocyte Transformation Tests

Frozen-stored lymphocytes (3, 4) from healthy persons were used The cultures, containing 200 000 responder lymphocytes in 0 5 ml of TC 199 medium with Hepes buffer, heparin antibiotics and 20% AB serum were stimulated with phytohemagglutinin (PHA [illegible] purified protein derivate [illegible] 000 I U / [illegible] vay mixed [illegible] ting cells lymphocyte culture (MLC), [illegible] irradiated with 2000 rad (a total of 400 000 cells per 0 5 ml) Following incubation for seven days including ^{14}C thymidine incorporation for 20 hours the cultures were harvested with a Skatron multiple cell culture harvester (3, 4) The results of scintillation counting were expressed as disintegrations per minute (dpm) Cultures were shown in triplicate or quadruplicate

HEAC Rosettes (B lymphocytes)

Human type A erythrocytes were washed thrice with Hanks' solution and 1 ml of a 2 5% cell suspension was incubated for 30 min at 37° C with rabbit anti-A serum (1 2500) and then with fresh mouse serum (100 µl) as complement source Three ml of Hanks' solution was added and the suspension was mixed gently Rosettes were formed by mixing the lymphocyte suspension (100 µl) with the sensitized erythrocyte suspension (100 µl) for 5 min at room temperature and the number of rosettes formed by 200 lymphocytes were determined

E Rosettes (T Lymphocytes)

100 µl of a 0 5% suspension of unsensitized sheep erythrocytes (SRBC) in Hanks' solution were incubated for 30 min at 37° C with 100 µl lymphocyte suspension and 20 µl human AB serum, pre absorbed with SRBCs for 18 hours at 4° C The mixture was centrifuged for 5 min at 40 *g* at room temperature and incubated for 18 hours at 4° C After gentle mixing the number of rosettes formed by 200 lymphocytes was determined in a counting chamber Lymphocytes with ≥3, 2 and 1 erythrocyte were counted separately For further details see (5)

Incubation of Lymphocytes with PZ-protein Preparations

Protein preparations containing 0 8–2 2 mg PZ protein/ml were used The total protein concentration of PZ preparations and control preparations from male serum was the same In the final cell suspensions protein was diluted to half of the given values Lymphocytes were incubated with protein preparations either before testing or simultaneously with testing In some cases the lymphocytes were washed after incubation before the tests were performed The lymphocytes were incubated at 37° C in an atmosphere containing 5% CO_2 and 95% humidity

Leucocyte Migration Inhibition Tests

The agarose technique described by *Bendixen & Søborg* (2) and by *Clausen* (7) with direct PPD (tuberculin purified protein derivative)-induced migration inhibition was used Heparinised blood was mixed with 5% dextran (Dextran 250 Pharmacia) dissolved in 0 15 M NaCl solution in a volume ratio of 4 1 After sedimentation for 1 hour at 37° C the leucocyte rich plasma was centrifuged for 5 min at 150 g The sediment was washed three times with Hanks solution (5 min, 150 *g*) and re suspended in medium TC-199 (Flow Laboratories) The cell suspension was adjusted to 2 1 × 10^8 cells per ml and incubated for 30 min at 37° C in 2% CO_2 with 100 µg PPD/ml (50 000 I U /ml Statens Seruminstitut Copenhagen)

The agarose medium consisted of TC-199 containing 2% agarose (Litex Glostrup, Denmark) 10% horse serum and penicillin streptomycin (66 I U /ml and [illegible] addition of 66µg/ml The pH was adju[illegible]

$NaHCO_3$ The agarose layers (5 mm thickness) were incubated at 37° C for 1½ hours in 2% CO_2 in air Wells (2 3 mm) were punched in the gel and 7 µl aliquots of both antigen incubated suspensions and control suspensions containing 1 5 × 10^6 leucocytes were added The gels were incubated at 37° C in 2% CO_2 and 95% humidity for 18 hours The area of migration was projected onto paper of uniform quality and measured by cutting and weighing the paper image Migration indices (MI) were calculated from quadruplicate tests as ratios of migration areas in antigen-containing cultures to migration areas in control cultures without antigen

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• Variation of incubation time with comparison between 3 hours pre incubation with PZ protein preparations before stimulation and addition of PZ protein simultaneously with stimulation did not produce significantly differing results Nor did washing the cells after pre incubation

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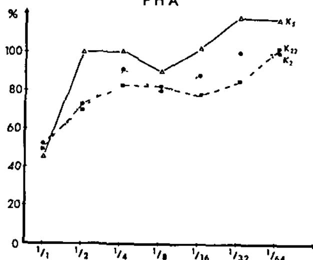
Effect of PZ protein Preparations and Control Preparations on Leucocyte Migration Inhibition

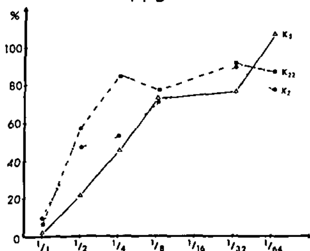
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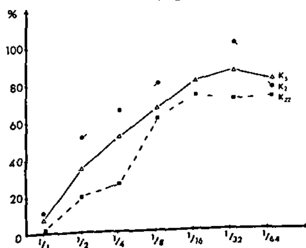
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MS_2	7 408 ± 147	259 ± 70	3 035 ± 199
PS_2	87 ± 9	32 ± 3	94 ± 10

Lymphocytes were pre incubated with PZ-protein (PS_2 1 0 mg PZ-protein/ml) and the control preparation (MS_2) for 3 hours Normal test, test performed on lymphocytes without prior incubation with protein preparations Results are given in dpm (means ± 1 SD) of triplicate analyses







with control preparations The preparations were incubated with the leucocytes for 30 minutes before/simultaneously with/or after PPD incubation As expected incubation with PPD inhibited leucocyte migration whereas incubation with PZ protein or control preparations did not (Table 3) Incubation with PZ protein preparations annulled PPD induced migration inhibition for all the forms of incubation tried and the migration indices obtained were significantly ($p<0.01$) different from migration indices obtained using PPD alone Incubation with control preparations did not produce this effect (Table 3)

DISCUSSION

PZ protein has been studied in the last few years espcially by *Damber et al* (9) and by von *Schoultz et al* (16) and *Bohn* (6) and *Stimson* (13) They have shown that PZ protein concentration increases during pregnancy and reaches a maximum in the third trimester, that the serum concentration is about 1000 µg/ml at parturition (15) and that the concentration 6 weeks after parturition falls to a level of 1–40 µg/ml serum

The partly purified PZ protein preparations used in this study inhibited blast transformation of T lymphocytes after stimulation with PHA PPD and in MLC with a clear dose response effect in the PPD and MLC tests This inhibition was obtained using PZ protein concentrations of 5 to 400 µg/ml that is corresponding to or much lower than those found *in vivo* during late pregnancy Inhibition developed after incubation with PZ protein and the cells did not regain responsiveness after conventional washing

The weaker inhibitory effect exerted by control preparations from male and female non pregnancy sera was probably due to the presence of small quantities of PZ protein (1–40 µg/ml serum according to *Damber et al* (9)) and/or other immunosuppressive non PZ proteins (8 11) The

Fig 1 Dose dependent effect of PZ protein preparation on a) PHA induced lymphocyte transformation b) PPD induced lymphocyte transformation and c) after stimulation with histoincompatible allogene cells in MLC

Abscissa Dilutions of PZ protein 1/1 corresponds to a final concentration in the cultures of 375 µg PZ protein/ml

Ordinate Disintegrations per minute in per cent of control response

K_5 K_{21} and K_{22} are three different lymphocyte populations

TABLE 2 *Rosette Formation by Untreated Lymphocytes and Lymphocytes Incubated with PZ protein (1 5 mg/ml) Preparations and Control Preparations with and without Washing after Incubation*

	PZ protein					Control				
	B	T			Null cells	B	T			Null cells
		>3	2	1			>3	2	1	
mal Test	20 5 ± 2 9	61 0c ± 10 0	4 8 ± 1 0	8 3 ± 4 4	5 5e ± 2 4	20 5 ± 2 9	61 0 ± 10 0	4 8 ± 1 0	8 3 ± 4 4	5 5k ± 2 4
90 a + wash	18 8 ± 1 0	38 0d ± 2 0	5 8 ± 0 5	15 0 ± 3 8	19 0f ± 0 8	19 0 ± 0 8	51 0i ± 12 1	2 3 ± 1 5	9 3 ± 1 5	18 5l ± 12 3
90 a − wash	17 8 ± 1 3	39 3 ± 12 9	4 8 ± 1 0	11 5 ± 2 9	24 3g ± 1 9	18 5 ± 1 9	49 5 ± 10 5	2 3 ± 1 3	9 5 ± 4 4	20 3m ± 6 2
whole testb	19 0 ± 0 8	41 0 ± 11 0	5 5 ± 0 6	8 0 ± 2 9	26 5h ± 8 6	19 5 ± 1 3	48 0 ± 7 3	3 8 ± 0 5	7 2 ± 1 4	21 5n ± 7 0

a) Pre incubation for 90 minutes before testing
b) Incubation simultaneous with testing
c vs d) $p < 0\ 05$ e vs f,g h) $p < 0\ 05$ i vs j) n s k vs l) n s k vs m n) $p < 0\ 05$ d vs j) n s
Counts refer to B and T rosettes (≥3 2 1 SRBC/lymphocyte) formed/200 lymphocytes × 0 5 Results are given as means ± 1 SD of four separate experiments Normal test tests on untreated lymphocytes

TABLE 3 *Effect of PZ protein and Control Preparations on Leucocyte Migration Inhibition*

Antigen	Group 1	Antigen	Group 2
PPD	0 87 ± 0 06	PPD	0 91 ± 0 03
PZ	0 96 ± 0 04	Control	1 02 ± 0 12
PPD ± PZa	1 08 ± 0 08d	PPD ± controla	0 90 ± 0 04
PPD ± PZb	1 05 ± 0 06d	PPD ± controlb	0 92 ± 0 07
PZ ± PPDc	1 01 ± 0 04d	Control ± PPDc	0 95 ± 0 07

Results are given as migration indices (MI) − ratio of migration areas in antigen-containing cultures to migration areas in cultures devoid of PPD
Group 1 means ± 1 SD of 8 experiments
Group 2 means ± 1 SD of 4 experiment PZ
a)
b)
c)
d)

marked difference in the inhibition exerted by MS and PS preparations e g in the PHA and MLC-tests in Table 1 can not be due to a toxic effect of the PS preparation as the two preparations were prepared in parallel by identical methods

Protein fractions containing PZ protein have previously been shown to inhibit lymphocyte transformation after stim lat

prepared using salt precipitation and observed a dose response effect similar to the one reported here In the present study at a concentration of 375 µg PZ protein/ml PPD and MLC responses were completely depressed but the effect on the PHA response was less pronounced (Fig 1)

PHA Con A and PPD are

number of HEAC rosettes was unaffected by incubation of lymphocytes with PZ protein while a tendency for T lymphocytes to bind fewer SRBCs was observed The effect on T lymphocytes could not be removed by conventional washing *Stimson* (13) observed a reduction in the number of E rosettes with ≥3 SRBCs following incubation of lymphocytes with PZ protein but the effect on E rosettes with fewer SRBCs HEAC rosettes or null cells was not reported

In the leucocyte migration inhibition studies

described here PZ protein annulled PPD induced migration inhibition at a concentration of 250 μg/ml whereas such an abrogating effect was not obtained with corresponding preparations from male serum *Stimson* (13) observed a blocking of leucocyte migration inhibition when PZ-protein was incorporated in the culture medium In our study a clear blocking effect was demonstrated also when PZ protein was added 30 min after PPD stimulation and thus in the system used seemed to be due to an influence on release or function of leucocyte migration inhibition factors (LIF) rather than a receptor blocking

The present investigation and *Stimson s in vitro* studies as well as the recent study of the effect of PZ protein in an *in vivo* transplantation model (17) suggest that this pregnancy associated protein represents one of the humoral factors that can exercise an immunosuppressive effect during pregnancy

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FRACTIONATION OF CONNECTIVE-TISSUE-ACTIVATING FACTORS FROM THE CULTURE MEDIUM OF SILICA-TREATED MACROPHAGES

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Aalto M & Kulonen E Fractionation of connective tissue activating factors from the culture medium of silica treated macrophages Acta path microbiol scand Sect C 87 241–250 1979

The medium of cultured SiO_2-treated peritoneal macrophages contained a factor which enhances the incorporation of labelled proline to collagen and other proteins in granulation tissue slices cells and polysomes Simultaneously the activity of alkaline RNase in the whole medium was decreased in comparison with the corresponding control Polyvinylpyridine N-oxide PVNO protected the macrophages against SiO_2 Latex particles and *E coli* lipopolysaccharide decreased the RNase activity in the macrophage medium but unlike SiO_2 did not cause liberation of the collagen synthesis-stimulating factor Fractionation of the medium by gel filtration chromatography showed the SiO_2-pretreatment to have caused a very significant decrease in the aggregation state of RNase The fraction from gel filtration chromatography that contained the SiO_2 liberated factor stimulating collagen synthesis also contained the *disaggregated* RNase There was no RNase activity in the control sample A homogenous protein (mol wt 14300) was isolated with repeated gel filtrations from the medium of silica treated macrophages It increased the incorporation of ^3H proline and ^3H thymidine into cultured *granuloma* cells

Key words Collagen synthesis DNA synthesis fibrosis macrophages, silica

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In an earlier report from our laboratory we have demonstrated a SiO_2-liberated soluble fibrogenic factor in homogenized peritoneal macrophages (4) The increased collagen synthesis has been shown in slices and the cell free polysomal system (5) from

synthesis *in vitro* The sources of both the macrophages and the fibroblasts differ in the various studies

In the present work we have resorted to the macrophage culture because it is not necessary to break the macrophages and the procedure is more relevant to the conditions *in vivo* The use of cultured fibroblasts from the granulation tissue is superior to slices from the same tissue because the experiments can be longer in duration and the target cells are homogenous

The preliminary gel filtration chromatography of

forms of RNase (1)

MATERIAL AND METHODS

Cultivation of macrophages The peritoneal macrophages of male rats were washed out with sterile 0 9% NaCl solution containing 10 U/ml heparin The

land) always buffered with 20 mM Hepes (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid, Sigma, St Louis, Mo , U S A) and *supplemented with 100 IU* penicillin and 50 µg streptomycin/ml (Orion, Finland) The cells were washed twice in this medium and thereafter placed in disposable Nunclon® N1460 or N1470 cell culture flasks (Nunc Products, Roskilde, Denmark) 3–4 × 10^6 cells/ml *of medium The flasks* were incubated for 2 h at 37° C in an atmosphere of 5% CO_2 + 95% air (the standard gas phase) The nonadherent cells were rinsed out with the medium The new media were added to the cultures with or without 0 25 mg of silica (Dorentrup quartz DQ 12 <5 µm) *per ml* In some experiments the macrophages were pretreated with latex particles (Dow-Latex, Ø 1 091 µm, Serva, Heidelberg, Germany) or lipopolysaccharide W (*E coli* 055 B5, Difco Laboratories, Detroit, Mich , U S A) instead of SiO_2 The media were renewed every second day without further addition of SiO_2 The media from the cultures were centrifuged at 20 000 *g* for 30 min at 4° C and the pH adjusted to 7 4 The supernatants were stored at −20° C before use

Macrophages were also cultured in the presence of 10% foetal calf serum (FCS, No 29-101-54, 4-055M, Flow Laboratories Ltd) in order to check the requirement of serum The procedure was carried out as described above The media from cultures of different ages were centrifuged at 20 000 *g* for 30 min and both the effect of supernatants on the cell-free protein synthesis and the RNase activity were assessed

Alkaline ribonuclease activity was determined according to Liu et al (22)

Where indicated, polyvinylpyridine-N-oxide (PVNO, [illegible]

Cultivation of granulation tissue cells The granulomas were induced in 2-month-old Wistar rats by the implantation of 0 8 × 1 × 2-cm viscose cellulose sponges (Kongsfoss Fabrikker A/S Oslo 2, Norway) (30) After two weeks *the granulomas were harvested into cold* 0 9% NaCl-solution, sliced and cut with scissors into small pieces, and suspended in the Dulbecco-Hepes (20 mM) medium containing antibiotics as listed above After being washed twice by filtration through sterile cheese *cloth, the* pieces were again suspended into Dulbecco-Hepes medium, now containing also 0 05% trypsin (Sigma, Type III) and 0 1% collagenase (Sigma, Type I) The suspension was incubated at room temperature for 1 h while being stirred vigorously by a *magnetic* stirrer The detached cells were filtered through cheese cloth and washed twice with the medium without the enzymes The cells were placed into the cell culture flasks and grown in the *Dulbecco-Hepes* medium [illegible]

from the third [illegible] incorporation experiments

Fractionation of the macrophage medium The media from 3-4-day-old cultures of SiO_2-treated and nontreated *macrophages* were applied on a Sephadex G 100 (Pharmacia, Uppsala, Sweden) column (2 3 × 23 cm) eluted with Krebs-Ringer-Hepes buffer (3) Samples of 3 *ml were collected* and the absorbances at 280 nm measured The samples were then pooled into three fractions according to protein concentrations (I II and III, Fig 2) The effects of the samples and the pooled fractions on ^3H proline incorporation into granuloma *tissue polysomes, slices and cultured granuloma cells* were tested as described below

Fractions III (300 ml) were concentrated to 2 ml in an Amicon ultrafiltration cell No 52 with UM2 membrane (Amicon Corp , Lexington, Mass 02173, U S A) *washed with 250 ml distilled water, applied on the* Sephadex G-25 (superfine) column (Fig 4) and eluted with distilled water Absorbance at 230 nm was measured The fractions were pooled, lyophilized and tested for ^3H proline and ^3H thymidine incorporation in *cultured granuloma cells*

Incorporation of ^3H proline into granulation tissue slices The 20 000 *g* supernatants from macrophage cultures in the Dulbecco-Hepes medium were incubated with granulation tissue slices (3 ml/500 mg wet weight) *Cold proline 2 87 mM was present (3) After 15 min* preincubation at 37° C in a shaking incubator, 10 µCi ^3H proline (TRA 82, The Radiochemical Centre Amersham, England) was added The 3 h incubation was stopped by placing the flasks on crushed ice The slices *were homogenized in an Ultra-Turrax homogenizer* (Janke & Kunkel, Staufen i Breisgau, Germany) washed six times with 80% ethanol and finally once with diethyl ether

The dry homogenates were weighed and hydrolysed in 6 N HCl for 3 h at 130° C The hydrolysates were evaporated on a boiling water bath The total and hydroxyproline radioactivities were measured according to *Juva & Prockop* (18)

Incorporation of ^3H proline into cultured granuloma cells The confluent granuloma cell cultures were trypsinized diluted 1 4 with the modified Dulbecco-Hepes medium with 10% FCS and divided among cell culture tubes (*Nunclon® N1409) 1 5 × 10^5 cells/2 ml* medium No exogenous proline was added The medium in all tubes was changed every day The ^3H proline incorporation experiments were carried out with cultures of different ages (1–5 days, Fig 1) The supernatants *from macrophage cultures were added on granuloma cell* layers with 5 µCi ^3H proline and 10% FCS and incubated for a further 24 h at 5% CO_2 + 95% air The media and the cells were separated and *dialysed against* tap water for 3 days The total and hydroxyproline radioactivities were measured according to *Dehm & Prockop* (12)

When the fibrogenic effects of pooled Sephadex *G-100 fractions I, II and III* were tested with confluent cultured granuloma cells, 10% FCS, 22 4 mM glucose and 10 µCi ^3H proline were added to the Nunclon® N 1409 tubes Otherwise the procedure was as above The Amicon-concentrated and lyophilized fractions III were dissolved in Dulbecco-Hepes medium with antibiotics and 10% FCS and then added to the confluent granuloma cell layer Incorporation with ^3H proline was

measured as above from the cells and the medium separately

When cells were incubated with fraction B from the Sephadex G 25 superfine column only total ^3H proline radioactivity was measured because of the small sample The cells were grown in wells of a cell culture plate (Nunclon® Micro Test Plate) with growth area of 0 3 cm^2 to the confluent state in Dulbecco-Hepes medium with the antibiotics and 10% foetal calf serum The culture media were replaced by the new media where the fraction B was dissolved 0 2 µCi ^3H proline was added to each well and incubation was carried out overnight. After incubation, the medium was pipetted separately from each well into a test tube with 2 ml 5 N PCA The tubes were kept in the cold overnight. The precipitates were collected on glass fibre discs (Whatman GF/A) and washed with cold 5 N PCA The radioactivity of the discs was counted in 5 ml PPO Tergitol scintillation liquid (15 g PPO 50 mg POPOP 600 ml toluene and 400 ml Tergitol® Union Carbide 15 S 9 supplied by Turun Saippua Oy Turku Finland) The cells were detached with 0 25% trypsin and collected on glass fibre discs and washed with 0 9 % NaCl using an automatic cell harvester The discs were dried at room temperature and their radioactivity counted with 5 ml of scintillation liquid (1 g PPO 3 3 mg POPOP in one litre of toluene) in the Packard Liquid Scintillation Spectrometer

Incorporation of ^3H thymidine The granuloma cells were grown in the wells of the cell culture plate to the confluent state as above and then incubated with the medium containing the dissolved fraction B and 0 20 µCi of ^3H thymidine (TRK 300 *methyl* ^3H thymidine 5 µCi/mmol The Radiochemical Centre Amersham England) for 24 h The media were discarded and the

[illegible]

Cell free protein synthesis Polysomes from rat granulation tissue were separated according to *Palmiter* (26) and the 30 S supernatant from wheat germs was obtained according to *Roberts & Paterson* (28) The standard reaction mixture for cell free protein synthesis has been [illegible]

[illegible]

supernatant and 50 µl of the sample to be assessed The mixture was incubated at 37° C for 90 min The charged tRNAs were deacylated by the addition of 1 M tris HCl buffer pH 10 0 and incubated for a further 10 min The tubes were placed in crushed ice and 2 ml 5% (w/v) TCA was added The precipitated protein was collected by filtration through discs of glass fibre sheet and washed with 20 ml cold 5% TCA The radioactivity of protein on the glass fibre discs was measured in PPO Tergitol scintillation liquid

Incorporation of ^{14}C amino acids to macrophages On the third day of macrophage culture after occasional pretreatment with SiO_2 2 5–5 0 µCi ^{14}C protein hydrolyzate (CFB 25 The Radiochemical Centre Amersham England) was added to 4–10 ml of fresh medium which was renewed every second day The cells were detached with a rubber policeman suspended in the Dulbecco Hepes medium and homogenized by repeated freezing and thawing The homogenate was centrifuged at 20 000 *g* and the protein precipitated and washed on a Millipore filter disc (Type GSWP 00010 0 22 µm) with 5% TCA (see below) The radioactivity of the filters was determined

Polyacrylamide gel electrophoresis was carried out according to *Lehtinen et al* (21) Molecular weight markers (mol wt. range 14 300–71 500 BDH Chemicals Poole England) were used as standards

RESULTS

After the treatment with SiO_2 the macrophages secreted in the culture media substances which stimulated the incorporation of ^3H proline in cell free protein synthesis in polysomes from granulation tissue (Table 1) The stimulatory effects varied with the time factors In the first 48 h media from culture incubated with SiO_2 there were not yet significant differences between control and SiO_2-treated samples as regards the effect on protein synthesis The relative stimulation was maximal in media from 3–4 days of culture after which it levelled off

It was shown by preliminary experiments that a 48 h period was more favourable for the pretreatment with SiO_2 than 24 h or 72 h periods Smaller concentration of SiO_2 (100 mg/l) was without effect. The polysome preparations are variable which accounts for the variations between the experiments

SiO_2 decreased RNase [illegible]

[illegible]

difference between control and SiO_2-treated macrophages The addition of 0 1 per cent polyvinylpyridine N-oxide PVNO with SiO_2 cancelled the inhibitory effect of SiO_2 on the RNase activities

The addition of 10% FCS to the macrophage culture had no effect on the liberation of the fibrogenic factor The stimulation by SiO_2-pretreatment with and without serum was equally about +20% The serum itself contained very high RNase activity (data not presented) which did not affect the action of SiO_2

When macrophages were treated with SiO_2 [illegible]

[illegible] media were much greater from control than from SiO_2-treated macrophages The age of the

land) always buffered with 20 mM Hepes (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid, Sigma, St Louis, Mo, USA) and supplemented with 100 IU penicillin and 50 µg streptomycin/ml (Orion, Finland) The cells were washed twice in this medium and thereafter placed in disposable Nunclon® N1460 or N1470 cell culture flasks (Nunc Products, Roskilde, Denmark) 3–4 × 10⁶ cells/ml of medium The flasks were incubated for 2 h at 37° C in an atmosphere of 5% CO_2 + 95% air (the standard gas phase) The nonadherent cells were rinsed out with the medium The new media were added to the cultures with or without 0 25 mg of silica (Dorentrup quartz DQ 12 <5 µm) per ml In some experiments the macrophages were pretreated with latex particles (Dow-Latex, Ø 1 091 µm, Serva, Heidelberg, Germany) or lipopolysaccharide W (*E coli* 055 B5, Difco Laboratories, Detroit, Mich, USA) instead of SiO_2 The media were renewed every second day without further addition of SiO_2 The media from the cultures were centrifuged at 20 000 *g* for 30 min at 4° C and the pH adjusted to 7 4 The supernatants were stored at −20° C before use

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Alkaline ribonuclease activity was determined according to *Liu et al* (22)

Where indicated, polyvinylpyridine-N-oxide (PVNO, Bay No 3504 Substanz, lyophilisiert, Bayer AG, Werk Elberfeld, Wuppertal, Germany) was added with SiO_2 to the macrophage culture at a final concentration of 0 1%

Cultivation of granulation tissue cells The granulomas were induced in 2-month old Wistar rats by the implantation of 0 8 × 1 × 2-cm viscose-cellulose sponges (Kongsfoss Fabrikker A/S, Oslo 2, Norway) (30) After two weeks the granulomas were harvested into cold 0 9% NaCl solution, sliced and cut with scissors into small pieces, and suspended in the Dulbecco-Hepes (20 mM) medium containing antibiotics as listed above After being washed twice by filtration through sterile cheese cloth, the pieces were again suspended into Dulbecco-Hepes medium, now containing also 0 05% trypsin (Sigma, Type III) and 0 1% collagenase (Sigma, Type I) The suspension was incubated at room temperature for 1 h while being stirred vigorously by a magnetic stirrer The detached cells were filtered through cheese cloth and washed twice with the medium without the enzymes The cells were placed into the cell culture flasks and grown in the Dulbecco Hepes medium containing the antibiotics plus 10% FCS to the confluent state The media were renewed every third day The cells from the third to the tenth passage were used for the incorporation experiments

Fractionation of the macrophage medium The media from 3-4-day-old cultures of SiO_2-treated and nontreated macrophages were applied on a Sephadex G-100 (Pharmacia, Uppsala, Sweden) column (2 3 × 23 cm) eluted with Krebs-Ringer-Hepes buffer (3) Samples of 3 ml were collected and the absorbances at 280 nm measured The samples were then pooled into three fractions according to protein concentrations (I II and III, Fig 2) The effects of the samples and the pooled fractions on ^3H proline incorporation into granuloma tissue polysomes, slices and cultured granuloma cells were tested as described below

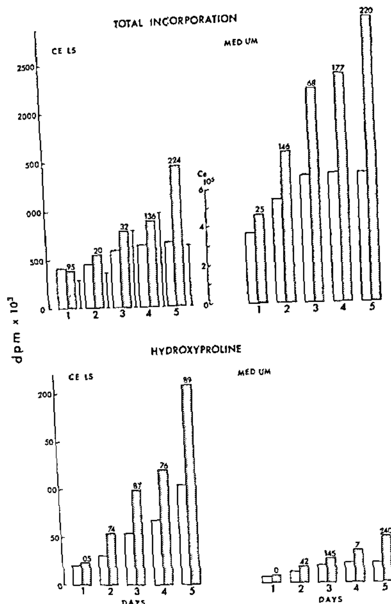
Fractions III (300 ml) were concentrated to 2 ml in an Amicon ultrafiltration cell No 52 with UM2 membrane (Amicon Corp, Lexington, Mass 02173, USA) washed with 250 ml distilled water, applied on the Sephadex G-25 (superfine) column (Fig 4) and eluted with distilled water Absorbance at 230 nm was measured The fractions were pooled, lyophilized and tested for ^3H proline and ^3H thymidine incorporation in cultured granuloma cells

Incorporation of ^3H proline into granulation tissue slices The 20 000 *g* supernatants from macrophage cultures in the Dulbecco-Hepes medium were incubated with granulation tissue slices (3 ml/500 mg wet weight) Cold proline 2 87 mM was present (3) After 15 min preincubation at 37° C in a shaking incubator, 10 µCi ^3H proline (TRA 82, The Radiochemical Centre Amersham, England) was added The 3 h incubation was stopped by placing the flasks on crushed ice The slices were homogenized in an Ultra-Turrax homogenizer (Janke & Kunkel, Staufen i Breisgau, Germany) washed six times with 80% ethanol and finally once with diethyl ether

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F g 1 Effects of the med a from control ☐ and S O treated ▩ macrophage cultures (from 3–4 days) on the ^{3}H p o ne ncorpora on nto granula on t ssue f broblasts cultured for 1 5 days separately into total prote n and hyd oxyp o ne both in the ce s and n the med um The number of cells s shown by the vert cal bars At the top of the co umns s shown the percentage effect of S O The con rol s taken to be 100 0

cultures had no great effect. The add tion of 0 1% PVNO w th S O_2 presumably prevented the rupture of macrophages and the effect on the protein synthes s was highly stimulatory (106–366%) This effect was much higher than in the respective control samples with either PVNO or S O_2 a fact which remains unexplained

Granuloma cells were incubated with unfractionated macrophage media (3–4 days) at different phases of the growth in culture (Fig 1) The stimulating effect of SiO_2-treated macrophages on the ^{3}H proline incorporation increases steadily with the growth At the confluent stage the stimulation is about two-fold both in cells and media. Stimulation

TABLE 1 *Effect of Macrophage Culture Media on the Cell Free Synthesis of Proteins by Granulation Tissue Polysomes with Reference to SiO_2-Pretreatment and RNase Activity of the Media*

Age of cells in culture	Medium time with cells	Exp no	Protein synthesis cell free[b] c p m						RNase ΔA_{260} h⁻¹ml⁻¹			
			Control[a]	SiO_2-treated	*n*	P	Effect of SiO_2 %[d]		Control	SiO_2-treated	Effect of SiO_2 %	
2 days[c]	0–2 days with SiO_2	1	322 ± 75	358 ± 44	6	NS	111		32	30	93	
		2	561 ± 22	472 ± 36	6	0 05	84	mean	40	36	90	mean
		3	1065 ± 91	1180 ± 88	6	NS	110	111 %	28	27	96	84%
		4	299 ± 5	414 ± 52	3	NS	139	(NS)	67	39	58	(NS)
		4 PVNO	405 ± 4	326 ± 4	3	NS	80		58	65		
4 days	3–4 days without SiO_2	1	387 ± 29	932 ± 79	6	0 001	240		13	4	30	
		2	753 ± 78	1315 ± 71	6	0 001	174	mean	12	8	67	mean
		3	978 ± 39	1511 ± 211	3	0 05	154	171 %	11	1	9	31%
		4	613 ± 60	707 ± 60	3	NS	115	(P < 0 05)	23	4	17	(P < 0 0
		4 PVNO	480 ± 37	869 ± 149	3	0 05	181		24	27	105	
6 days	5–6 days without SiO_2	3	1684 ± 72	1425 ± 196	3	NS	84		0	0	–	
		4	1009 ± 45	1627 ± 99	3	0 01	161		0	0	–	
		4 PVNO	1018 ± 100	1184 ± 142	3	NS	116		16	24	149	
8 days	7–8 days without SiO_2	3	1202 ± 127	1325 ± 53	3	NS	110		–	–	–	

The figures give the means ± S E M

[a] Macrophages treated similarly but without SiO_2

[b] Granulation tissue polysomes and wheat germ extract in the presence of media from SiO_2 treated and control macrophages (5)

[c] SiO_2 was added only for the first 48 h period

[d] SiO_2 treated/control

TABLE 2 *Effect of Addition of SiO_2 Particles on the Protein Synthesis in Cultured Macrophages*

Age of culture	Exp no	^{14}C Amino acids incorporated c p m		
		Control	SiO_2 treated	%[b]
Media				
3–4 days[a] (with ^{14}C Amino acids)	3	17 430	1795	10
	5	7115	2170	31
	5 PVNO	7100	33 100	466
5–6 days[a]	3	7700	1140	15
	5	7740	465	6
	5 PVNO	4070	8385	206
7 days[a]	3	2010	59	3
	5	2337	299	13
	5 PVNO	974	2106	216
Homogenized cells	6			
7 days[a] supernate		979	379	39
sediment		1094	259	24
	6 PVNO			
7 days[a] supernate		629	259	41
sediment		486	598	123

The numbers of the experiments refer to the same experiments as in Table 1

[a] SiO_2 was added only for the first 48 h period and the radioactive amino acids only at the beginning of the 48 96 h period

[b] SiO_2 [illegible] d/control

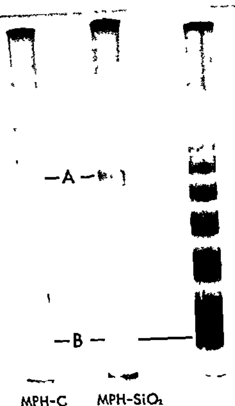


Fig 4 Gel electrophoresis patterns of the concentrated

/1 500 on the right Letters A and B refer to Fig 5

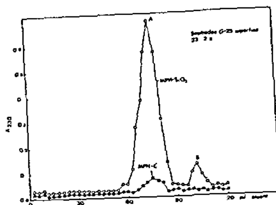


Fig 5 The repeated gel filtration of the concentrated fraction III (Sephadex G 100 Fig 2) from control ● and SiO_2-treated O macrophage culture media with Sephadex G 25 superfine in distilled water Cf Fig 4

When the macrophage culture media (3–4 days) were chromatographed on the Sephadex G 100 column and eluted with Krebs Ringer Hepes buffer, only one large protein peak (fraction II) could be detected at 280 nm (Fig 2)

The fibrogenic effects of the pooled fractions (I, II and III) of SiO_2-treated and control macrophage media from the Sephadex G-100 column were tested by the incubation of these fractions with granuloma slices and ^3H proline Fraction III had the greatest stimulatory effect on the synthesis of collagen (Fig 3) Fractions I and II showed only slight or no stimulation on the protein synthesis in

compared with control macrophages (Table 3) The increase in hydroxyproline radioactivity in the culture medium as the effect of treatment with lipopolysaccharide can be disregarded as an experimental variation

(data not shown) where the stimulation by fraction III was about 30% The ^3H proline and ^3H thymidine incorporations were also significantly increased (Table 4) when the granuloma cells were

TABLE 4 *Effect of the Purified Fibrogenic Factor from Macrophage Culture Media on the ^3H Proline and ^3H Thymidine Incorporations into Granuloma Cells*

Source of factor[a]	^3H Proline cpm cells	^3H Proline cpm medium	^3H Thymidine cpm	Protein μg[b]	RNase E/ml/h[b]
Control macrophage	2600 ± 169	688 ± 19	1900 ± 85	18	0
SiO_2-treated macrophages	3227 ± 165*	1134 ± 59**	2550 ± 184**	110	0 64
Dulbecco medium	2227 ± 260	1007 ± 25	3228 ± 209	–	–

Mean ± SEM is given n = 4 The statistically significant differences as compared with the controls are indicated as *($p < 0.01$) and **($p < 0.0025$) evaluated by the *t* test

[a] Cells were incubated with fraction B from Sephadex G 25 superfine column (Fig 4)

[b] Protein and RNase originate from the same amount of macrophages control and SiO_2 treated macrophage culture media are treated equally

TABLE 3 *Comparison of Effects of Culture Media from SiO_2-, Latex- or Lipopolysaccharide-treated Macrophage on ^3H Proline Incorporation into Cultured Granuloma Cells*

Culture and addition	RNase $\Delta A_{260}h^{-1}ml^{-1}$	Hydroxyproline c p m Cells	Hydroxyproline c p m Medium	Total incorporation c p m Cells	Total incorporation c p m Medium
Control[a]	19 (100%)	4260 (100%)	16 060 (100%)	351 300 (100%)	201 720 (100%)
SiO_2 0 25 mg/ml	10 (52%)	5280 (124%)	19 390 (120%)	474 570 (135%)	243 600 (120%)
Latex 500 µg/ml	5 (28%)	3800 (89%)	14 730 (92%)	338 390 (96%)	207 380 (102%)
Lipopolysaccharide 50 µg/ml	8 (39%)	4260 (100%)	19 750 (123%)	362 620 (103%)	163 700 (81%)

The figures are the means of duplicate experiments

[a] with untreated macrophages 100%

of the synthesis of total protein and of collagen is the same

The effect of SiO_2 on macrophages was compared with the effects of latex particles and *E coli* lipopolysaccharide There was no increase in ^3H proline incorporation into cultured granuloma cells when incubated with 3-4-day-old media from latex- or lipopolysaccharide-treated macrophage cultures The decreases in the RNase activities of media were about 70% with latex, 60% with lipopolysaccharide and 50% with SiO_2 treated macrophages

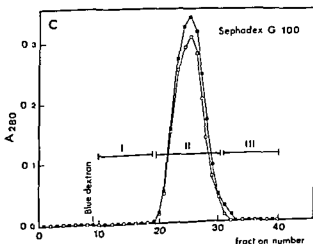


Fig 2 Gel filtration of the culture media from control ... Sephadex G 100 ... ictions referred to in the legends of Fig...

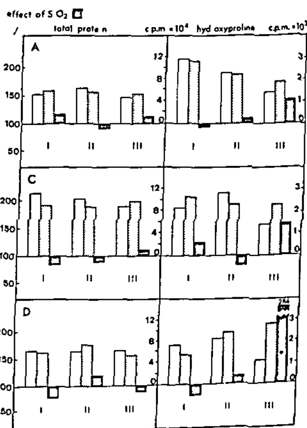


Fig 3 Effects of the pooled Sephadex G 100 gel filtration fractions I II and III of the media from control ▢ and SiO_2 treated ▣ macrophages on the synthesis of collagen (hydroxyproline) and total proteins in the granulation tissue slices The percentage effects of SiO_2 treatment are represented by the third columns ■ in each group The letters A C and D refer to the same experiments as in Fig 6

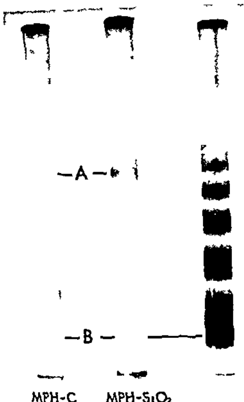


Fig 4 Gel electrophoresis patterns of the concentrated fraction III (Seph [illegible]

[illegible] 100 on the right Letters A and B refer to Fig 5

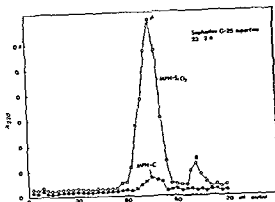


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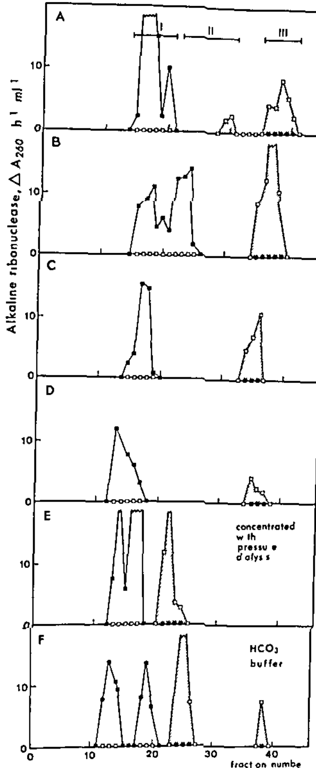


Fig 6 Gel filtration distribution of alkaline RNase activity secreted into the medium on 3rd and 4th days by ■ d (□ haded

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Differences were observed between the elution patterns of RNase in the media of control and SiO_2-treated macrophages (Fig 6) The RNase activity of media of SiO_2 treated macrophages was eluted noticeably later than those of control macrophage media

DISCUSSION

Preparations of SiO_2-treated macrophages The macrophage culture medium is a cleaner source of SiO_2 liberated factors than the extract of the subcellular particles of macrophages The medium which contains the cell secretions related to phagocytosis of SiO_2 can be lyophilized and stored in frozen state Gel filtration with Sephadex G 100 removes the bulk of the proteins and fraction III is a suitable starting point for further purification Both the gel filtration patterns and the gel electrophoresis patterns of the concentrated fraction III from control and silica treated macrophages with Sephadex G 25 superfine are different We think that the protein B which is lacking in the control samples is a fibrogenic factor

The collection of rat peritoneal macrophages is tedious and should be replaced by procurement of macrophages from other sources e g the phagocytizing monocytes of the »buffy coat« of human blood (17) or by transformed macrophage like cell lines which could be grown *in vitro* We know from preliminary experiments that the human »buffy coat« cells can replace the rat peritoneal macrophages (2) The fibrogenic factor of rat at least can be tested with fibroblasts from other species e g chicks (15)

Fibroblast systems In this work we have applied several systems of fibroblasts 1) granulation tissue slices which contain the cells in their natural environment This system has limited viability and access of extracellular materials to the cells is restricted 2) cultured granulation tissue cells which can be used in longer experiments and which respond with greater effect The effects on the secretion of fibroblasts can be tested by this system However only reparative fibroblasts can be used and the cells are liable to change 3) cell free protein synthesis with granulation tissue polysomes The

last method is rapid and economical in terms of reagents but the dispersion in the results tends to be great because the polysome preparations are vari able The most sensitive assay system in our hands has been culture of granulation tissue cells

Effect of SiO_2 on macrophages Our prime interest in this work has been the fibrogenic, *i e* the collagen synthesis-stimulating effect of the prepara tion from SiO_2-treated macrophages We conside-red it to be a model for experimental silicosis but also for pathological fibrosis in general

Bec ... ho ... f ... S O

tive preparations from control macrophages we suggested that the fibrogenic effect was due directly to a decreased RNase activity (6) RNase activity of macrophages is indeed decreased as the effect of the SiO_2-treatment and the state of aggregation of the RNase activity (cf 24) is very markedly changed, but collagen synthesis stimulating activity is not always associated with lower RNase activity than the controls as can be seen from Table 4 The discrepancies may be solved by consideration of (*i*) the various time courses and (*ii*) the actual levels of specific RNase activities This finding leads to two main questions What is the role of RNase in experimental silicosis and what is the true mecha nism of the SiO_2-liberated fibrogenic factor if not the stabilization of polysomes in the fibroblasts by inhibition of RNase either in macrophages or fibroblasts?

Other effects of SiO_2 on the liberation of lysosomal enzymes from the macrophages are well known from the work of several authors (7 11 16) In general lysosomal enzymes are released on the activation of macroph ... (22 ...

... y of macrophages in terms of protein synthesis Phagocytosis in rat Kupffer ... have

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Preparative work on the various biological factors (see 29) from the culture medium of normal and SiO_2-pretreated macrophages is required to solve the topical questions, for example, the relationship of specific RNase and collagen synthe sis stimulating activities, and, above all to identify the biological factors in molecular terms

We are greatly indebted to Professor *A G Heppleston* Edinburgh for encouragement, criticism and advice Institutional grants from the *Finnish Medical Research Council* and the *Sigrid Jusélius Foundation* are gratefully acknowledged

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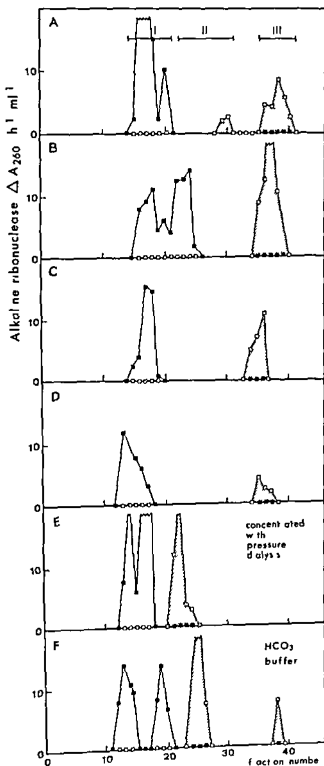


Fig 6 Gel filtration distribution of alkaline RNase activity secreted into the medium on 3rd and 4th days by control (■ open areas) and silica treated (□ shaded areas) macrophages Sephadex G 100 column eluted with Krebs Ringer Hepes buffer pH 7 4 Letters A F refer to the different experiments and I III to the fractions shown in Fig 2

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EXAMINATION OF PAROTID SALIVA FOR ANTIBODIES REACTING WITH *STREPTOCOCCUS MUTANS*, LIPOTEICHOIC ACID AND PEPTIDOGLYCAN BY THE ENZYME-LINKED IMMUNOSORBENT ASSAY

DOUGLAS BRATTHALL, ANETTE CARLÉN KENNETH W KNOX and ANTHONY J WICKEN

Faculty of Odontology University of Goteborg S 400 33 Goteborg 33 Sweden Institute of Dental Research United Dental Hospital Sydney N S W 2010 Australia and School of Microbiology University of New South Wales Kensington N S W 2033 Australia

Bratthall D Carlén A Knox K W & Wicken A J Examination of parotid saliva for antibodies reacting with *Streptococcus mutans* lipoteichoic acid and peptidoglycan by the enzyme linked immunosorbent assay Acta path microbiol scand Sect C 87 251–255 1979

... Six salivas were tested against peptidoglycan but these tests indicated only low levels of antibodies Absorption of saliva with whole cells of *S mutans* inhibited the homologous reaction by up to 87% and the reaction with LTA by up to 52% Also prior treatment of saliva with LTA caused a decrease in the salivary IgA reaction with LTA and with whole cells of *S mutans* Addition of peptidoglycan to saliva did not markedly affect the salivary IgA reaction with *S mutans* *The data show that LTA may be responsible* for part of the salivary IgA reaction with whole cells of *S mutans* The *significance of LTA* in this reaction may vary between different subjects and for different serotypes of *S mutans*

Key words Parotid saliva antibodies *Streptococcus mutans* lipoteichoic acid peptidoglycan enzyme linked immunosorbent assay

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Received 27 xi 78 Accepted 30 i 79

The recognition that *Streptococ*... there has been considerable interest in an immunological approach ... dental caries experience (2 8) These results have generally failed to provide substantial evidence for a definitive correlation between the level of antibody present and the amount of dental caries although the flow rate of parotid saliva may be important (8) Interest in salivary antibodies *particularly* IgA derives from their possible protective effects which most likely would depend on their ability to prevent bacterial cell adherence (10 21)

There have also been numerous animal experiments based on the immunological prevention of dental caries (2) and two recent studies have clearly *demonstrated a* correlation between decreased dental caries and increased salivary IgA on inoculation of *the experimental* animals with whole cells of *S mutans* (16) or immunogenic components (21)

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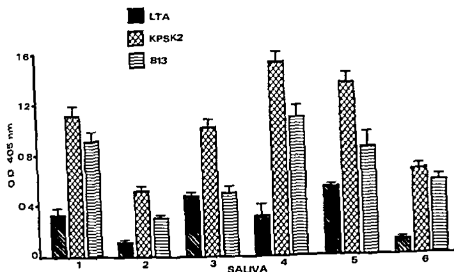


Fig *1* ELISA values of saliva from six subjects tested against lipoteichoic acid (LTA) and *S mutans* strains KPSK2 (c) and B 13 (d)

S D is indicated in the diagram (only half extension shown) Controls total procedure with saliva included but without any coating of the tubes 0 08 ± 0 03, tubes without saliva but coated with LTA 0 04 ± 0 02 with KPSK2 0 06 ± 0 00 and with B13 0 05 ± 0 00

of lipoteichoic acid, and then unconjugated anti IgA was added to one set of four tubes, the remaining tubes served as controls The subsequent addition of enzymeconjugated anti IgA to all tubes showed that the prior treatment with anti-IgA caused a 50% decrease in the amount of reaction

Cross absorption of antibodies reacting with S mutans *and lipoteichoic acid* The ability of cell suspensions of *S mutans* KPSK2 to remove salivary antibodies reacting with homologous cells and lipoteichoic acid was examined To 5 ml undiluted heated and centrifuged saliva was added, washed and pack [illegible]

respectively when the bacteria were suspended in 25 ml phosphate buffered saline After shaking at room temperature for 60 min the saliva samples were centrifuged diluted ½ and their reactivity compared with that for unabsorbed saliva. The homologous reaction with KPSK2 cells was inhibited 86% and 87% by cells of O D = 2 and O D = 5 respectively, the respective values for the inhibition of the reaction with lipoteichoic acid were 45% and 12%

Conversely two saliva samples were examined for the inhibition by lipoteichoic acid of the reaction with strain KPSK2 and lipoteichoic acid Saliva samples (3 ml) were shaken for 90 min at room temperature with 0 3 ml lipoteichoic acid with a concentration of 1, 10 or 100 µg/ml or with 0 3 ml phosphate buffered saline (control) The results (Fig 2) showed that prior treatment of saliva with lipoteichoic acid, particularly at 100 µg/ml, caused the expected decrease in the subsequent ELISA reaction with lipoteichoic acid There was also a lesser though significant decrease in the reaction of the absorbed saliva with cells of KPSK2

Detection of antibodies reacting with peptidoglycan The saliva of six subjects were studied for antibodies reacting with peptidoglycan, lipoteichoic acid and *S mutans* cells The results showed again that reactions were obtained with lipoteichoic acid and bacteria but the ELISA values with peptidoglycan were low For four of the subjects the ELISA values were only twice as high, or less than the control readings (without any coating of the ELISA tubes) and in the other two subjects the results were about three times these values

Cross-absorptions employing peptidoglycan at a final concentration in saliva of 10 or 100 µg/ml were performed as described above Absorption of saliva with strain KPSK2 did not decrease the reaction with peptidoglycan any further, nor did addition of peptidoglycan to saliva significantly affect the saliva reaction with strain KPSK2 in any of six samples tested

In such animal experiments the source of the immunogenic stimulus is known However, with respect to the studies on the antibody levels in human sera and saliva there is the difficulty, which is frequently overlooked of knowing whether the antibodies reacting with a particular organism are specific or cross reactive and produced against another organism Earlier studies (15) showed that many of the observations on human serum antibodies could have been influenced by the presence of cross reactive antibodies to the lipotei choic acid component Lipoteichoic acid occurs as a surface antigen in many gram positive bacteria where its »backbone« of polyglycerol phosphate accounts for reports of a common antigen in these organisms (12, 24)

This study was aimed at determining whether such antibodies to lipoteichoic acid could also be detected *in parotid saliva and could therefore* account for part of the reactivety of saliva with whole cells of *S mutans* In addition saliva has been examined for the presence of antibodies reacting with peptidoglycan because of its occurrence as major cell wall component of all bacteria and because it contains common antigenic determinants in both the carbohydrate and peptide moieties (18)

MATERIAL AND METHODS

Bacterial strains S mutans strains KPSK2 (serotype *c*) and B13 (se

night at 37°

to contain 2

Before use the cells were washed once in phosphate buffered (pH 7 1 0 01 M) saline

Lipoteichoic acid Lipoteichoic acid was extracted from cells of *Lactobacillus casei* NCTC 6375 with hot water and purified as previously described for the component from *Lactobacillus fermentum* (22) The lipoteichoic acid from this organism has a polyglycerol phosphate component devoid of sugar substituents and

Peptidoglycan The wall fraction of *L casei* NCTC H831 was isolated following mechanical disruption of cells and the polysaccharide separated from the pepti doglycan by dilute acid hydrolysis (11) Analyses of the purified peptidoglycan used in these studies have been reported previously (11) The peptidoglycan 2 mg/ml was sonicated for 20 minutes at 20 kHz centrifuged at 18 000 × *g* for 60 minutes and the supernatant employed in the serological studies

Collection of saliva For methodological studies parotid saliva from one male and two females was obtained in ice-chilled tubes using Curby cups The secretions were slightly stimulated with citric acid In further studies two groups with six females in each age 18–30 were used as saliva donors The citric acid stimulation was adjusted *to result in about 6 ml of* parotid saliva in 20 minutes After collection the salivas were immediately frozen and stored at −20° C until the analysis

Enzyme linked immunosorbent assay The enzyme-linked immunosorbent assay (ELISA) of *Engvall & Perlmann* (9) including some modifications of this technique proposed by *Bratthall et al* (4) was adapted to measure the salivary IgA antibodies Polyethylene tubes (11 × 55 mm Heger Plastics AB Stockholm Sweden) were coated with lipoteichoic acid peptidoglycan or bacteria dissolved or suspended in phosphate buffered (pH 7 1 0 01 M) saline with 0 02% NaN_3 The preparations 0 5 ml in each tube were placed in a shaking water bath of 37° C for 3 h and then overnight at 4° C All tests were performed with tubes in quadruplicate The optimal coating concentration accor ding to *Bratthall et al* (4) were used for the bacteria For lipoteichoic acid and peptidoglycan the optimal concen tration was shown to be 1 µg/ml and 100 µg/ml respectively After rinsing the tubes 0 5 ml saliva was added to each tube The saliva samples had been thawed heated at 56° C for 30 min centrifuged at 18 000 × *g* for 20 min and diluted twofold in phosphate buffered saline containing 0 05% Tween 20 After 5 h at room temperature 0 5 ml anti immunoglobulin (anti IgA α

Tween 20 was added After overnight incubation the amount of conjugate bound was determined by the addition of p nitrophenylphosphate (Sigma) which is a substrate for the alkaline phosphatase Appropriate *washings between the different steps were included* according to the original procedure The absorbance was read with a Beckman DB GT spectrophotometer and the results expressed as

$$\text{absorbance} \times \frac{100}{t}$$

where ›t‹ is the number of minutes when the reaction was stopped by the addition of NaOH Controls included the total procedure without any coating of the tubes or addition of buffer *instead of saliva to coated tubes*

RESULTS

Comparison of reactions of salivary antibodies with lipoteichoic acid and S mutans Parotid salivas from six females were examined *for their reactivity* in tubes coated with a) 1 µg/ml of lipoteichoic acid b) cells of *S mutans* KPSK2 and c) cells of *S mutans* B13 The results in Fig 1 show the mean value and the Standard Deviation and indicate that saliva may contain quite a significant level of antibody reacti vity with lipoteichoic acid

To confirm that serological reactivity was *due to* immunoglobulin A, twofold diluted saliva was added to eight *tubes previously coated* with 1 µg/ml

added significance as the studies by *Rolla et al* have led to the proposal (17) that the phosphate groupings of teichoic acids play an important role in adherence

D B and A C acknowledge the support of the *Swedish Medical Research Council* Project No 4548 K W K and A J W acknowledge the support of the *Australian National Health and Medical Research Council*

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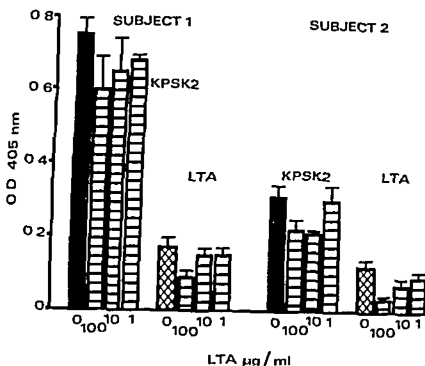


Fig 2 Effect of addition of different concentrations of lipoteichoic acid (LTA) to saliva prior to ELISA tests with *S mutans* strain KPSK2 and with LTA

DISCUSSION

The application of the ELISA technique for the detection of salivary IgA antibodies reacting with *S mutans* strains has confirmed previous observations where such antibodies were detected by the agglutination reaction (1, 3, 14, 19) The ELISA method does have the advantage, however, of greater sensitivity and also greater accuracy so that it enables a better comparison of the levels of antibody between individuals It should however be pointed out that longitudinal studies have shown individual variation in the salivary concentration of antibodies reacting with oral streptococci (3) Variability for an individual can also be shown if the flow rate and the volume of collected saliva are not kept constant (4, 20) For this reason approximately 6 ml of saliva was collected under a standardized period of time, 20 minutes

A comparison of the results obtained for six individuals shows that in each case the ELISA-value for strain KPSK2 of serotype *c* was greater

reacting with lipoteichoic acid The reaction of antibodies to lipoteichoic acid with strain KPSK2 was indicated by the lower values when two saliva samples were first absorbed with lipoteichoic acid Although the reduction for absorbed saliva is not great, its significance is increased by the observation that the addition of lipoteichoic acid to saliva only partially decreased the subsequent reaction of the absorbed saliva with lipoteichoic acid

Another potential cross-reacting antigen is peptidoglycan (18) The saliva samples reacted only poorly with peptidoglycan from *L casei* however, and cross-absorption studies indicated that such antibodies, if present, do not contribute to the reactivity of saliva with strain KPSK2

Serotype *c* strains are frequently the most prevalent amongst *S mutans* strains in plaque where they can be detected with specific antibodies (5) Cross reactions between *S mutans* strains of different serotypes do occur (6), however, and it has been shown that rabbit antiserum to *S mutans* strains, including KPSK2 and B13 contain cross-reactive antibodies to the lipoteichoic component (13) These antibodies are specific for the glycerol phosphate chain (23, 24), and the results therefore suggest that part of the reaction of *S mutans* strains with salivary IgA is due to the presence of such antibodies

Although the presence of cross reacting antibodies means that the organism inducing the antibody response can not be defined, such antibodies could nevertheless have a potentially beneficial role On the assumption that secretory IgA antibodies can prevent an organism's adherence to oral tissues including tooth enamel (10), such cross-reacting antibodies would have the potential to prevent adherence of a variety of bacteria The detection of antibodies reacting with lipoteichoic acid may have

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DEMONSTRATION OF THE NON-IDENTITY BETWEEN THE Fc RECEPTOR FOR HUMAN IgG FROM GROUP A STREPTOCOCCI TYPE 15 AND M PROTEIN, PEPTIDOGLYCAN AND THE GROUP SPECIFIC CARBOHYDRATE

POUL CHRISTENSEN* ANDERS GRUBB** RUNE GRUBB* GUNILLA SAMUELSSON* CLAËS SCHALÉN* and MAJ LIS SVENSSON*

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Christensen P Grubb A Grubb R Samuelsson G Schalén C & Svensson M L. Demonstration of the non identity between the Fc receptor for human IgG from group A streptococci type 15 and M protein peptidoglycan and the group specific carbohydrate Acta path microbiol scand Sect C 87 257-261 1979

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Key words Streptococci M protein peptidoglycan group A carbohydrate human IgG

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[illegible] antibodies The agglutination caused by the extracts was shown

DEMONSTRATION OF THE NON-IDENTITY BETWEEN THE Fc RECEPTOR FOR HUMAN IgG FROM GROUP A STREPTOCOCCI TYPE 15 AND M PROTEIN, PEPTIDOGLYCAN AND THE GROUP SPECIFIC CARBOHYDRATE

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Key words Streptococci M protein peptidoglycan, group A carbohydrate human IgG

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Hot hydrochloric acid extract ad modum Lancefield of type 15 group A streptococci is capable of precipitating IgG in a majority of human sera, although type 15 infections are reported to be uncommon (10 12) Preliminary investigations have suggested that the precipitation could be caused by streptococcal IgG Fc receptors in the extract (10) The frequency of precipitation varies between populations which indicates a restricted specificity for human IgG (10) It has hitherto not proved possible to separate M protein from the [illegible]

to be due to an interaction between the extract and the Fc part of IgG (2)

We now describe that the Fc receptor of type 15 group A streptococci can be separated from M protein peptidoglycan and group specific carbohydrate by subjecting *alkaline* extracts of the bacteria to agarose gel electrophoresis

MATERIALS AND METHODS

Streptococcal Strains

The following reference strains of group A streptococci were used M 1 (8198) M 8 (8324) M 15 (100070) and the Griffith strain T 44 (Henson glossy)

Streptococcal Extracts

Group A streptococci type 15 were cultured in 20 litres of Todd Hewitt broth and harvested as described previously (10) The bacteria were washed in 0 15 M NaCl suspended in 100 ml 0 15 M NaCl and the pH was raised to 10 0 by adding 2 M NaOH The suspension was heated in a boiling water bath for 10 min and then immediately cooled on ice The pH was adjusted to 7 0 with 2 M HCl the suspension centrifuged at 3000 *g* for 15 min and the supernatant used as alkaline extract

Alkaline extracts of group A streptococci types 1 8 and T 44 were prepared from overnight cultures in 300 ml Todd Hewitt broth The amounts of 0 15 M NaCl NaOH and HCl used were reduced in proportion to the lesser volume of broth used

... the alkal ne extract of type 15 ...

minutes at 37° C the trypsinization was stopped by addition of soy bean trypsin inhibitor (Sigma type 1–s) to 2 mg/ml

Peptidoglycan was prepared from M 1 group A streptococci with hot formamide as described previously (9)

Human Sera IgG IgG Fragments and M Components

Sera were collected from 23 apparently healthy individuals among the laboratory staff heated at 56° C ...

described earlier (2) ... (batch no 58803) was purchased from AB Kabi (Sweden) and used unless otherwise indicated at a concentration of 40 mg/ml phosphate buffered saline (0 12 M NaCl 0 05 M phosphate pH 7 2)

The purified human myeloma proteins were identical with those used previously (2) 5 IgG1 M components 2 IgG2 4 IgG3 3 IgG4 1 IgD and 3 IgM Furthermore one IgA M-component was purified as described earlier (2)

Electrophoretic Techniques

In the standard procedure for demonstration of precipitation lines between streptococcal extract and human serum the streptococcal extract components were first separated by electrophoresis and the separated extract then tested against the serum by diffusion Twenty ml 0 6 % agarose (Miles Ltd England) in 0 075 M barbital buffer pH 8 6 containing 2 mM calcium lactate was poured over a 205 × 110 mm glass plate and circular wells taking 8 µl extract cut 75 mm from the projected anode After application of the extract a voltage of 200 V was applied for 45 min unless otherwise stated A 2 mm broad channel 90 mm long was cut 3 mm from the application well in the direction of the current Two hundred µl serum was applied and the diffusion was allowed to proceed for 2 days at room temperature

Preparatory electrophoresis was performed in a 4 mm layer of 1% agarose in the barbital lactate buffer The extract was applied in a 6 mm broad channel 190 mm long cut in the agarose gel 75 mm from the projected anode A voltage of 150 V was applied for 2 hours after which the gel was cut into 5 mm wide slices After freezing the pieces overnight at −80° C followed by thawing and centrifugation at 3000 *g* protein containing supernatants were harvested The fractions were concentrated four times on a concentration cell (B15 Amicon corp Mass U S A)

Electromigration experiments were performed in barbital lactate buffer by 6 V/cm for 16 h with an agarose concentration of 1% (w/v) and 1 5 mg commercial polyclonal human IgG/ml gel

The precipitates were stained with Coomassie Brilliant Blue

Rabbit Anti Streptococcal Sera

Rabbits were immunized intravenously with a suspension of washed and heat killed streptococci as described (8) Four animals were immunized with type 15 group A streptococci and two each with types 1 8 and T 44 streptococci Anti group A C and G rabbit sera were purchased from Difco (Mich U S A)

Agglutination Tests with Ri-coated human red cells were performed as described previously (10)

RESULTS

Precipitation Patterns Observed in Diffusion Experiments Involving Electrophoretically Separated Alkaline Extract of Type 15 Group A Streptococci and Human Sera

After electrophoresis of the extract three main precipitation archs were obtained in diffusion against commercial human polyclonal IgG (Fig 1) an anodal line (»1«) a double arch line extended along the well in which the extract was applied (»3«) and an elongated line situated near the serum well extending from the cathodal side of the extract

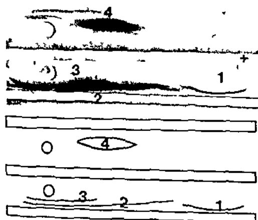


Fig 1 Precipitation lines obtained in diffusion experiments involving electrophoretically separated alkaline extract of type 15 group A streptococci and human commercial polyclonal IgG or rabbit anti type 15 serum Upper trough rabbit anti type 15 serum Lower trough commercial polyclonal IgG middle trough saline The round application wells for the alkaline extract are shown to the left 1 Line 1 2 line 2 3 line 3 and 4 line 4 (see text) + indicates the anode

application well to the cathodal start point of line 1 (»2«)

Nineteen of 23 sera (83%) from apparently healthy individuals gave line 3 while 6 of them (26%) gave line 1 Line 2 was obtained with 2 sera only (9%) Lines 1 and 2 were *not obtained* with any of the sera without the presence of line 3 Four of the sera (17%) did not give any line

Fifteen µl of one of the sera giving both line 1 and line 3 (serum SM) was added to 5 µl of the alkaline extract of type 15 and the mixture then subjected to electrophoresis After diffusion against 5 sera giving both line 1 and line 3 with unabsorbed extract, no precipitation lines corresponding to 1 and 3 were obtained

A few sera (2–6) gave precipitates corresponding to line 3 against the separated extracts of types 1, 8 and T 44 group A streptococci but precipitates corresponding to line 1 were not seen Line 2 was not obtained with types 1 8 and T 44 extracts

Separation of the Streptococcal Components Responsible for the Three Precipitates with Human Sera

In the following experiments the results obtained refer to tests with commercial human polyclonal IgG and a human serum giving both line 1 and line 3 (serum SM)

Lines 1 and 3 disappeared, while line 2 was unaltered after trypsinization of the type 15 alkaline extract Line 2 could be inhibited by addition of peptidoglycan to the commercial human IgG or serum SM

Line 1 was obtained with the fractions precipitated at 20–30% ammonium sulphate saturation of the extract, while line 3 was obtained with the fractions prepared by 40% saturation

Serum SM and commercial human IgG were absorbed with Cowan I staphylococci as described (1) After absorption an essentially unaltered line 3 was observed, while line 1 had disappeared

The electrophoretic fractions of the extract giving lines 1 and 3 were cut out of the agarose gel after electrophoresis Addition of the »line 1 -fractions« to SM or to commercial human IgG inhibited only the formation of line 1, but not line 3, while the »line 3 fractions« only suppressed line 3

Localization of the M-Protein and Group A Carbohydrate after Electrophoresis of the Alkaline Extract of Type 15 Streptococci

All four anti type 15 rabbit sera gave a line against the separated extract situated between the positions of line 1 and 3 observed with human sera (line 4 Fig 1) Line 4 was not obtained between the anti type 15 sera and the extracts of types 1, 8, and T 44 group A streptococci Rabbit antisera to types 1, 8 and T 44 did not give line 4 with the alkaline extract of type 15 Line 4 disappeared after trypsinization of the extract

Anti group A carbohydrate serum gave a precipitate against a non migrating component in the electrophoretically separated extracts of types 1, 8, 15, and T 44 This precipitate was not obtained with anti-C or anti G rabbit sera. The precipitates were obtained also after trypsinization of the extracts

Localization of the Streptococcal IgG Fc-Receptor after Electrophoresis of the Alkaline Extract of Type 15 Group A Streptococci

After electrophoresis of the extract, pieces of gel were cut out and investigated for content of agglutinating activity against Ri-coated human red cells Only the fractions surrounding the application well, corresponding to line 3 agglutinated the cells (titres 1 200–1 3200)

The alkaline extract was mixed with equal volumes of Fc fragments or Fab-fragments of pooled human IgG at concentrations of 0 6 – 2 5 mg/ml The mixture was subjected to electrophoresis and tested against serum SM and commercial human IgG in diffusion experiments On addition of Fc fragments to the extract, line 3 moved against the anode whereas Fab-fragments did not cause any change in line 3 (Fig 2)

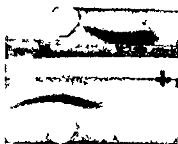


Fig 2 The effect of addition of Fc fragments of polyclonal IgG to alkaline extract of type 15 group A streptococci on the precipitation lines obtained between the electrophoretically separated extract and serum SM in diffusion experiments Trough serum SM upper well application well for equal volumes of Fc fragments and alkaline extract lower well application well for alkaline extract diluted 1 2 + indicates the anode

The alkaline extract mixed with Fc fragments as described above was subjected to electrophoresis and tested by diffusion experiments against rabbit anti group A carbohydrate serum The precipitate obtained did not change its character or position as compared with the results obtained with the extract alone

Characterization of Line 1

The streptococcal components responsible for line 1 were isolated by preparatory electrophoresis as described in »Methods« The preparation was then tested in electromigration experiments by applying it in a hole in an agarose gel in barbital lactate buffer containing commercial human polyclonal IgG which resulted in a »rocket« precipitate (Fig 3) Mixing the preparation with equal volumes of Fc fragments 0 6 – 2 5 mg/ml had no effect on the precipitate Mixing the preparation with the same amount of Fab fragments of pooled human IgG however resulted in depression of the precipitate (Fig 3)



Fig 3 Electromigration of fractions from preparatory electrophoresis corresponding to line 1 Agarose gel containing 1 5 mg commercial human polyclonal IgG/ml was used Left well fraction corresponding to line 1 diluted 1 2 Right well *fraction corresponding to line 1* mixed with an equal volume of Fab fragments (2 5 mg/ml) from human polyclonal IgG Middle well same as in the right well but Fab fragments 1 2 mg/ml were used

The preparation from the preparatory electrophoresis was also tested in the same system after mixing with 19 different purified human M-components at a concentration of 2 mg/ml No alteration of the precipitate obtained with commercial human IgG was found

DISCUSSION

After electrophoresis of the alkaline extract of type 15 group A streptococci it was possible to obtain strong precipitates in immunodiffusion experiments with sera from apparently healthy individuals One of the precipitation lines obtained line 3 was ascribed to a streptococcal Fc receptor for human IgG since the line could be displaced by addition of *Fc fragments but not Fab fragments of pooled* human IgG Fc fragments migrate towards the anode in electrophoresis performed under the conditions described here (3) Furthermore preparative electrophoresis of the alkaline extract showed that the fractions giving line 3 were capable of agglutinating IgG-coated human red cells at high titres Line 3 was obtained with 19 of 23 human sera 83% a proportion close to the 82% Swedes found to precipitate hot hydrochloric acid extract of type 15 in gel diffusion experiments (10)

Line 4 obtained in diffusion experiments involving electrophoretically separated alkaline type 15 extract and rabbit anti sera to type 15 group A streptococci was judged to contain streptococcal M protein It was only obtained with antisera to type 15 and not with antisera against 3 other types The antisera to type 15 did not give such a line with extracts of other streptococci Furthermore the component responsible for the precipitate in the alkaline extract was like M protein trypsin sensitive Since the components responsible for lines 3 and 4 had differing electrophoretic mobility it is clear that the streptococcal Fc receptor for human IgG in type 15 group A streptococci is separable from the precipitating type specific M protein

The streptococcal IgG Fc receptor could also be separated from the group A carbohydrate since the receptor was trypsin sensitive and could be displaced by electrophoresis after addition of human IgG Fc fragments in contrast to group A carbohydrate Also the Fc receptor was separable from peptidoglycan since line 3 was not suppressed by addition

of peptidoglycan to the commercial human IgG in contrast to line 2

Six of the 18 sera giving line 3 gave also another line line 1 against electrophoretically separated alkaline extract of type 15 group A streptococci Line 1 was different from line 3 since line 1 was suppressed in contrast to line 3 on absorption of SM serum or commercial human IgG with Cowan I staphylococci protein A of *S aureus* reacts with 3 of 4 human IgG subclasses (5) with 1 of 2 IgA subclasses (7) and with about 30 % of human IgM (4) In electromigration experiments the component giving line 1 was altered by addition of Fab-fragments but not Fc fragments of polyclonal human IgG in contrast to the component giving line 3 It seems possible that line 1 is produced by an antigen antibody precipitation since myeloma proteins did not influence the component producing the line The relation of the component giving line 1 to other streptococcal antigens – other than M protein peptidoglycan and the groupspecific carbohydrate – is still unknown Type 15 infections in humans are seldom met (12) in contrast to type 1 group A streptococcal infections for instance This makes the frequent occurrence of line 1 as compared with the absence of such precipitates with the other types difficult to understand at present The high protein concentration found in the type 15 extract as compared to the other extracts investigated might indicate that the type 15 extract could be »stronger« than the other preparations However the M1 M8 and T44 extracts concentrated 10 times did not give line 1 (unpublished observation)

The precise mechanism by which human IgG is precipitated by streptococcal Fc-receptors present in type 15 alkaline extract is also unknown Preliminary studies have shown that the Fc receptor also precipitates a number of purified human myeloma proteins The finding that line 3 was displaced rather than suppressed after mixing with Fc fragments might indicate that a larger part than the Fc part of an IgG molecule is involved in the precipitation phenomenon Furthermore the double arch shape of line 3 regularly found might indicate that two or more Fc receptors are present in the alkaline extract of type 15 group A streptococci Purification experiments involving the Fc receptor(s) in type 15 alkaline extract are in progress and might elucidate the mechanism of precipitation

This work was supported by the *Swedish Medical Research Council* projects no B79-13X-05196 and B79-16X-05211-01 and *Riksforbundet mot reumatism*

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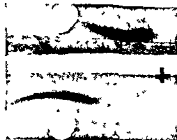


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The streptococcal IgG Fc receptor could also be separated from the group A carbohydrate, since the receptor was trypsin sensitive and could be displaced by electrophoresis after addition of human IgG Fc-fragments in contrast to group A carbohydrate Also, the Fc-receptor was separable from peptidoglycan, since line 3 was not suppressed by addition

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ELECTRON MICROSCOPY OF TREPONEMES SUBJECTED TO THE *TREPONEMA PALLIDUM* IMMOBILIZATION (TPI) TEST

II Immunoelectron Microscopy

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Hovind Hougen K Birch Andersen A & Nielsen H Aa Electron microscopy of treponemes *subjected to the Treponema pallidum immobilization (TPI) test II Immunoelectron microscopy* Acta path microbiol scand Sect. C 87 263–268 1979

The experiments were carried out in order to investigate whether human IgG globulin is adsorbed on to the surface of *T pallidum* cells during incubation with human syphilis serum in the *Treponema pallidum* immobilization (TPI) test Cells of *T pallidum* Nichols subjected to the TPI test were further incubated with ferritin conjugated rabbit antihuman IgG globulin Human IgG globulin could only be demonstrated on immunoimmobilized cells *i e* cells incubated with human syphilis serum and unheated guinea pig serum (GPS) The surface of the swollen cells was completely covered by a fuzzy layer on to which the ferritin molecules appeared to be attached In ultrathin sections of cells obtained from the same suspensions the surface of the outer cell membrane was outlined by ferritin molecules In these cells a rather wide gap was observed between the outer membrane and the cytoplasmic membrane The ribosomes seemed to have disappeared from the cytoplasm of the immunoimmobilized treponemes but were present in motile cells obtained from control incubations

Key words *Treponema pallidum Treponema pallidum* immobilization (TPI) test, immunoelectron microscopy immunoimmobilization.

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In our first paper of this series (3) we described a swelling of the cells of *Treponema pallidum* after incubation with serum from a syphilitic patient and unheated guinea pig serum (GPS) *i e* cells that were immobilized in the *Treponema pallidum* immobilization (TPI) test (4) It was suggested that specific antibodies in the human syphilis serum together with one or more factors present in unheated GPS reacted with receptor sites on the treponemal surface Previous studies (5) have shown that treponemes do not bind anti treponemal antibodies (immobilisin) even after prolonged incubation unless unheated GPS is also present. Similarly no antibodies were bound by organisms incubated with heated GPS and human syphilis serum (5)

In order to study these problems at the ultrastructural level we treated cells which had been incubated for the TPI test with ferritin conjugated rabbit antihuman IgG globulin The results of these experiments are given in this paper

MATERIAL AND METHODS

The incubation of the treponemes were carried out as described and tabulated in Table 1 of the previous paper (3) The incubation time used was 8 hours since by then cells incubated with serum from a syphilitic patient (Cop 111) (5) plus fresh unheated GPS were all immobilized

Acta path microbiol scand Sect C 87 263-268 1979

ELECTRON MICROSCOPY OF TREPONEMES SUBJECTED TO THE *TREPONEMA PALLIDUM* IMMOBILIZATION (TPI) TEST

II Immunoelectron Microscopy

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Hovind Hougen, K., Birch Andersen, A. & Nielsen, H. Aa. Electron microscopy of treponemes subjected to the *Treponema pallidum* immobilization (TPI) test. II. Immunoelectron microscopy. Acta path microbiol scand Sect C 87 263-268 1979

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Key words: *Treponema pallidum*; *Treponema pallidum* immobilization (TPI) test; immunoelectron microscopy; immunoimmobilization.

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In our first paper of this series (3) we described a swelling of the cells of *Treponema pallidum* after incubation with serum from a syphilitic patient and unheated guinea pig serum (GPS), i.e. cells that were immobilized in the *Treponema pallidum* immobilization (TPI) test (4). It was suggested that specific antibodies in the human syphilis serum together with one or more factors present in unheated GPS reacted with receptor sites on the treponemal surface. Previous studies (5) have shown that treponemes do not bind anti treponemal antibodies (immobilisin) even after prolonged incubation unless unheated GPS is also present. Similarly no antibodies were bound by organisms incubated with heated GPS and human syphilis serum (5).

In order to study these problems at the ultrastructural level we treated cells which had been incubated for the TPI test with ferritin conjugated rabbit antihuman IgG globulin. The results of these experiments are given in this paper.

MATERIAL AND METHODS

The incubation of the treponemes were carried out as described and tabulated in Table 1 of the previous paper (3). The incubation time used was 8 hours since by then cells incubated with serum from a syphilitic patient (Cop 111) (5) plus fresh unheated GPS were all immobilized

whereas cells incubated with the same serum, but with heated GPS, were all motile

Each of the incubated samples was centrifuged at 15,000 g for 25 minutes at 4° C and the cells were washed by resuspension in two volumes of basal medium (5) After this, cell pellets were carefully suspended in 1 ml of a solution containing 0 5 ml ferritin conjugate*) plus 2 ml basal medium without albumin The suspended cells were kept in this mixture for 30 minutes at room temperature with intermittent shaking Then the cells were centrifuged at 15,000 g for 20 minutes at room temperature and washed in basal medium without albumin to reduce the amount of unspecifically adsorbed or trapped conjugate At this stage specimen grids with cells from each incubation mixture were prepared and stained by the multiple drop technique (2) 1 per cent ammonium molybdate, pH 7 2 was used as negative stain 1 5 per cent glutaraldehyde in SMC (0 3 per cent sucrose with 0 01 M $MgCl_2$ and 0 01 M $CaCl_2$) was added to the remaining cells in each tube The material was fixed for 30 minutes at room temperature, followed by continued fixation at 4° C for 9 hours After this prefixation the cells were centrifuged and the resulting pellet embedded in a few drops of 45° C warm melted 1 5 per cent Noble Agar (Difco) in SMC Small blocks (approximately 1 mm^3) were cut and postfixed for 1 hour at room temperature in 1 per cent OsO_4 in SMC with 10 per cent YAP medium (0 3 per cent yeast extract, 0 05 per cent sodium acetate and 0 3 per cent peptone medium, all Difco products) The blocks were impregnated for 1 hour with 2 per cent uranyl acetate in SMC before dehydration in alcohol and propylene oxide and embedding in Vestopal W

Ultrathin sections were obtained on the LKB ultrotome III microtome and were stained with magnesium uranyl acetate and lead citrate

Formvar coated carbon reinforced copper grids were used for negatively stained preparations as well as for the sections

Electron microscopy was performed as described previously (3) For this paper approximately 420 electron micrographs were studied

*) The ferritin conjugated IgG fraction of rabbit antihuman IgG heavy chain serum was obtained from Cappel Laboratories Downington, Pa USA The conjugate was controlled by immunoelectrophoretic analysis with various antisera prepared at this institute The antisera were rabbit anti horse ferritin swine anti-rabbit serum and swine anti rabbit IgG The product was found to contain an α_2 component which was seen with both anti ferritin and anti-rabbit IgG and also a faster moving component which was seen only with anti-ferritin The conjugate also contained precipitating antibodies against human IgG which was the only component seen when normal human serum was used as antigen in the well and the conjugate as antiserum in the trough The immuno electrophoretic analysis generally confirmed the specifications for the product given by the supplier i e some free ferritin was the main contaminant of the conjugated IgG rabbit antihuman globulin

RESULTS

Cells obtained from pellets of treponemes incubated with serum from a syphilitic patient together with unheated GPS will be referred to as immobilized cells and those incubated with the same serum but with heated GPS as motile cells Pellets of *T pallidum* were always contaminated with tissue debris from the rabbit testis

Negatively Stained Material

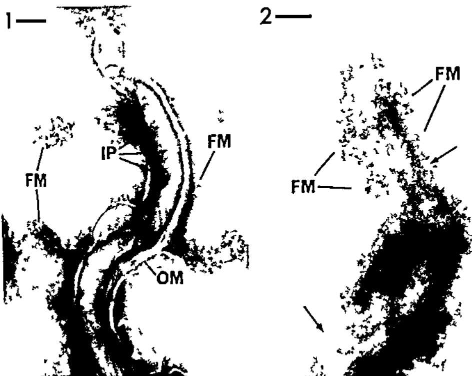
The morphology of the immobilized cells and the motile cells was described in the previous paper (3) The appearance of the motile cells was unaltered when such cells were further incubated with ferritin conjugated rabbit antihuman IgG globulin Even after the additional washings and centrifugations needed after this treatment, the treponemes presented the morphology of normal *T pallidum* cells Furthermore, practically no ferritin was present on the cell surface (Fig 1)

In contrast to this, immobilized cells that were incubated with the conjugate were swollen and the surface of their outer cell membrane was covered by the fuzzy material as well as with ferritin (Fig 2) The morphological details on these cells were often diffucult to resolve due to the amounts of ferritin molecules present on the cell surface, but on the whole the extra incubation with conjugate had very little influence on the general morphology of the immobilized cells (compare Fig 2 of this paper with Fig 3 in the previous paper (3)) It should be noted that some aggregates of ferritin were seen lying directly on the supporting film of the grid in the ferritin treated preparations of both motile and immobilized cells

Sectioned Material

The morphology of the sectioned motile cells appeared to be normal (1) The triple-layered outer membrane was found to tightly envelop the cells except at places where the flagella were seen in the space between the outer cell membrane and the cytoplasmic membrane (Fig 3) Very few or no ferritin molecules appeared to be attached to the outer membrane of the treponemes (Fig 3)

The appearance of the sectioned immobilized treponemal cells was very different from that of motile cells Immobilized cells were obviously swollen, and the exterior of their outer membrane was outlined by ferritin molecules (Figs 4 and 5 A, B) In some sections clusters of ferritin were observed in between treponemal cells These clusters were often shown to be attached to the surface of the outer membrane of cells included in an adjacent section Due to the swelling of the



All figures show cells of *T pallidum* Nichols subjected to the TPI test Figs 1 and 2 show material negatively stained with 1 per cent ammonium molybdate pH 7 2 and Figs 3 5 sectioned material GPS means guinea pig serum and ferritin conjugate stands for ferritin conjugated rabbit antihuman IgG globulin The same human syphilis serum (Cop 111) was used throughout The bar on each micrograph represents 100 nm

Fig 1 A treponeme incubated with syphilis serum and heated GPS prior to treatment with ferritin conjugate The cell shows a morphology corresponding to that of normal cells Clusters of ferritin molecules (FM) are scattered along the cell and are also present directly on the substrate of the grid The outer cell membrane (OM) tightly envelops the cytoplasmic body of the cell IP denotes the insertion points for the flagella (F) 90 000 ×

Fig 2 A treponeme incubated with syphilis serum and unheated GPS prior to treatment with ferritin conjugate The cell is swollen and the cell surface is covered with a fuzzy material and ferritin molecules (FM) 90 000 ×

periplasmic space of the immobilized cells a rather wide gap was present between the outer membrane and the cytoplasmic membrane in these cells (Figs 4 and 5 A B) The flagella were present in the space between the cell body and the outer membrane as is normally found However as a rule they were haphazardly distributed compared with their orderly arrangement within the more narrow space present in motile cells (cf Figs 3 and 5 A B) No abnormal intracellular structures were observed in the immobilized cells but it should be noted that the ribosomes were no longer distinguishable in the cytoplasm (Figs 4 and 5 A B) No ferritin was attached to the membranes of rabbit tissue origin present among the motile as well as the immobilized treponemes (Figs 3 and 4)

DISCUSSION

By the present techniques binding of human IgG globulin to cells of *T pallidum* could not be demonstrated following incubation of the cells with serum from a syphilitic patient (Cop 111) and heated GPS In contrast IgG was seen attached to the surface of the treponemes after incubation with the same serum when unheated GPS was present in the incubation mixture The results indicate that one or more thermolabile factors in the GPS are essential for the attachment of human antibodies (immobilisin) to the treponemal surface (*i e* for the sensitization) Complement in the GPS is probably involved but at present it is unknown whether or not the thermolabile factor(s) needed for the treponemal immobilization process are identical

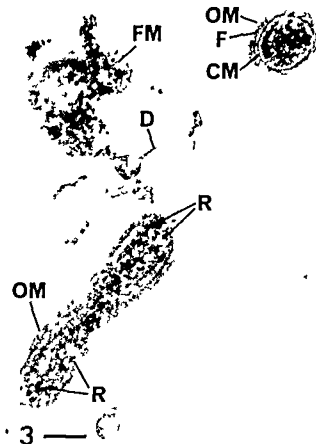


Fig 3 A section of treponemes which were treated with ferritin conjugate after incubation with syphilis serum and heated GPS The outer cell membranes (OM) tightly envelop the cells The flagella (F) are situated between the outer membrane (OM) and the cytoplasmic membrane (CM) R denotes ribosomes in the cell cytoplasm Aggregates of ferritin molecules (FM) are seen in between the treponemes and the rabbit tissue debris (D) 90 000 ×

with one or more of the thermolabile components of complement Whether these components are involved by reacting via the classical or alternate pathway cannot be decided from the present experiments Our experiments do however corroborate previous findings that the binding of antibodies to the treponemes was dependent on the presence of unheated GPS in the incubation mixture (5)

It has been discussed if immunoimmobilized cells of *T pallidum* should be considered dead or only paralysed (4) Our sectioned material shows that in immobilized cells ribosomes can no longer be seen in the cytoplasm The ribosomes may have been digested in autolytic processes and then the cells are most likely dead On the other hand they may also have been dissolved as a result of other reactions specifically directed towards the cell interior In any case ribosomes are present in motile cells after incubation with heated GPS and serum from a syphilitic patient for the same period of time so the immobilization process must have some effect also on the cell interior

Large aggregates of ferritin were found between cells in the preparations studied These aggregates probably originate from a surplus of human IgG present in our final suspensions The cells were only washed once before incubation with the ferritin conjugate in order to avoid too much damage of the cells during the manipulations and it is conceivable that unadsorbed IgG globulin was incompletely removed In an additional experiment we further washed the cells after incubation with the conjugate After three washings the amounts of unadsorbed ferritin between the treponemes were less pronounced while the washings had not removed the ferritin and thus the human IgG globulin from the cell surface

It should be mentioned that an occasional spheroid cell (6) was present in the cell suspensions studied Spheroid cells are non motile and no human IgG globulin could be demonstrated on the outer membrane of these cells (Fig 6) Furthermore the ribosomes were retained in their cytoplasm after the incubation which resulted in immobilization and loss of ribosomes in the other treponemes present We have also found a very low number of non motile helical treponemes in our suspensions before these were incubated with human syphilis serum and GPS When an unswol

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Fig 4 and Fig 5 A and B Sections of treponemes treated with ferritin conjugate after incubation with syphilis serum and unheated GPS The periplasmic spaces *are swollen (arrows) and the outer cell membranes* (OM) are outlined with ferritin molecules (FM) Note that no ribosomes are recognizable in the cell cytoplasm The flagella (F) in Figs 5 A and B are present in the wide gap between the outer membrane (OM) and the cytoplasmic membrane (CM) but less well ordered than in motile cells (cf Figs 5 A and 3) 90 000 ×

s for Figs 4 5 Note the
(FM) on the outer
the host tissue debris
noimmobilized cell

90 000 ×

FM
OM
FM
OM
CM
D
4

OM
F
CM
FM
5A

OM
F
CM
FM
FM
5B

FM
CM
D

OM
R
R
6

len cell was encountered among sectioned immobilized cells such a cell had ribosomes in the cytoplasm and did not show any ferritin *i e* any human IgG globulin on its surface We are tempted to identify the non motile cells with those that do not adsorb human IgG and our observations would then indicate that only motile cells of *T pallidum* are able to react with human IgG antibodies present in serum from syphilitic patients

We are indebted to *B Mansa* Department of Biophysics Statens Seruminstitut for carrying out the immunoelectrophoretic analysis of the conjugate and to *N Axelsen* Department of Treponematoses Statens Seruminstitut for valuable criticism of the manuscript We also wish to thank *A Knudsen* for her excellent assistance in the performance of the TPI tests *J Berg H Ravn* and *F Laursen* for their excellent assistance in electron microscopy and *A G Overgaard* and *F Laursen* for expert photographic work

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GENETIC CONTROL OF THE SPONTANEOUS HYPERTENSION IN THE NZB/Cr STRAIN OF MICE

Immunogenetic Considerations

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Svendsen U G Koch C M & Rubin B Genetic control of the spontaneous hypertension in the NZB/Cr strain of mice Immunogenetic considerations Acta path microbiol scand Sect C 87 269–273 1979

NZB/Cr mice spontaneously develop a high blood pressure This hypertension is developed during the first two months of age F1 hybrids between NZB/Cr and C57/Bl/6J (a normotensive mouse strain wh ...

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backcrosses and F2 hybrids gave a weak evidence that genes located in or closely linked to the H 2 complex do influence the spontaneously developed high blood pressure in the NZB/Cr strain of mice It is emphasized that further studies in larger populations of mice is necessary to establish the importance of linkage of genes to the H 2 complex for the spontaneous hypertension in the NZB/Cr strain of mice

Key words Spontaneous hypertension genetic control NZB/Cr mice immunogenetic considerations

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The New Zealand Black (NZB) strain of mice spontaneously develop autoimmune diseases including a hemolytic disease and a lymphoproliferative disorder (*Bielschowsky et al* 1959 *De Heer & Edgington* 1976 *Howie & Helyer* 1968) commencing after about 3 months of age Renal disease develops insideously also commencing after 3 to 6 months of age with intermittent proteinuria and cylindruria This disease remains clinically latent for the greater part of the life span although some animals develop a nephrotic syndrome with ascites hypoalbuminaemia and hypercholesterolaemia The nephropathy commences histologically with accumulation of hyaline material in the mesangium and the capillary basement membranes Aggregations of lymphoid cells are present around the smaller renal vessels and some small arteries show a necrotising arteritis (*Hicks & Burnet* 1966 *Holmes & Burnet* 1963 ...

It has been shown that the development of a marked increase in the blood pressure early in life precedes the vascular disease in the NZB/Cr strain of mice (*Svendsen* 1977) rendering the possibility of a pathogenic importance of the high blood pressure for at least some of the vascular disease probable ...

the treatment is given early on life before the vascular disease is apparent The thymus dependency of the high blood pressure in NZB/Cr mice thus suggests that the high blood pressure may be an autoimmune manifestation However, the influence of the incorporation of the nude alleles in the NZB/Cr genome is still only poorly understood then other explanations than the above mentioned is obviously possible

The mechanism of the genetic control of the development of the autoimmune disorders of the originally NZB/Bl strain of mice is by no means fully understood It is however, generally agreed at present that they are inherited as dominant traits and it is thought that a limited number of genes determine the autoimmune disorders (*Howie & Simpson* 1976)

The present study shows that also hypertension in the NZB/Cr mice is controlled by more than one gene The main influence on the blood pressure is exerted by gene(s) localized outside the major histocompatibility complex (MHC) However the results give some evidence that loci in or closely linked to the MHC do influence the level of blood pressure It is concluded that repeated studies in larger groups of backcross generations of mice are warranted to clarify this important question

MATERIALS AND METHODS

Animals Inbred SPF NZB/Cr (H2 d) and C57/Bl/6J [illegible]

Statens Seruminstitut were used

Systemic arterial blood pressures were recorded in the conscious mice In light ether anaesthesia a catheter was placed in the left carotid artery and connected to a *Tybjærg Hansen capacitance pressure transducer* (Simonsen & Weel Copenhagen) and a (G 14) graphic recorder (Danbridge Copenhagen) the blood pressure was subsequently recorded for one hour in the conscious semirestrained animal while it was placed in a *transparent plastic tube*

H 2 typing Two antisera were used in the present study A Balb/c anti C57/Bl/6J antiserum (H2 d anti H2 b) and a C57/Bl/6J anti Balb/c antiserum (H2 b anti H2 d) The antisera were obtained from mice which were immunized i p with (Balb/c x C57/Bl/6J) Fl spleen cells 8 times at weekly intervals They had cytotoxic titers of 1 256 against spleen or lymph node cells of relevant specificity *The cytotoxic assay was performed* as follows 0 2 ml of lymphoid cells (20×10^6/ml) was mixed with 0 2 ml of antiserum dilution (usually a 1 10 dilution in balanced salt solution = BSS) and the [illegible]ed for one hour at 4° C The reaction [illegible] discarded [illegible] of strain 2 guinea pig serum diluted 1 10 in BSS After incubation at *37° C* for 45 minutes the reaction tubes were added 0 5 ml of ice cold BSS They were placed on ice until counted for presence of live cells using trypan blue due exclusion

In every assay C57/Bl/6J Balb/c and (Balb/c x C57/Bl/6J) Fl cells were used as central cells and both antisera were used being internal controls for one another By means of the present assay the antisera killed *more than 90% of allogeneic cells* and less than 10% of syngeneic cells More than 90% of Fl cells were killed by both antisera Thus (NZB x C57/Bl/6J) Fl F2 or backcross animals were typed as *H2 d H2 b* or *H2 d* x H2 b on the basis of whether their spleen cells were killed by only H2 b anti H2 d or H2 d anti H2 b antiserum or by both antisera All cell suspensions were counted blindly

The investigations was performed in two parts

Part I

The genetic control of the inheritance of the high blood pressure in the NZB/Cr strain of mice The systemic arterial blood pressure was measured in 15 male NZB/Cr 15 male C57/Bl/6J and in 15 male Fl (C57/Bl/6J x NZB/Cr) mice 3 months old Subsequently blood pressure was measured in 3 months old male backcross hybrids between female Fl (C57/Bl/6J x NZB/Cr) mice and either C57/Bl/6J (n = 24) or NZB/Cr (n = 10) mice

Part II

The influence of the MHC (H 2) complex on the high blood pressure The possibility of linkage of the genes coding for the high blood pressure in the NZB strain of mice to the H2 complex was *investigated in backcross* hybrids (Fl (C57/Bl/6J x NZB/Cr) x C57/Bl/6J) (see above) and in 53 male 3 months old F2 hybrids bred from Fl (C57/Bl/6J x NZB/Cr) parent mice The influence of genes linked to the H2 complex for the high blood pressure was further investigated by comparing the blood pressure in two congenic strains of mice which only differed at the H 2 complex i e B 10 (H2 b) and B 10 D2 (H2 d) Such male mice had their blood pressure determined when they were 3 months old

For comparison of experimental results the students t test was used When this test implies a normally distributed population a »distribution free« test which does not assume that *the scores under analysis* were drawn from a normally distributed population were used as well (Mann Whitney U test) The five per cent level was used as indicative of significant differences All mean values are given with one standard deviation

RESULTS

The Genetic Control of the Inheritance of the High Blood Pressure in the NZB/Cr Strain of Mice

Fig 1 shows the blood pressure in the parental NZB/Cr and parental C57/Bl/6J mice and in Fl-hybrids between *the two parental strains* The blood

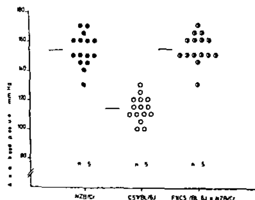


Fig 1 shows that the arterial blood pressure in both the NZB/Cr and the F1 (C57/B1/6J x NZB/Cr) strains of mice were significantly increased compared with the mean value observed in the C57/B1/6J strain of mice ($p < 0.001$) (● histocompatibility type dd ○ bb and ◑ bd) The solid line represents the mean value n = number of 3 months old male mice

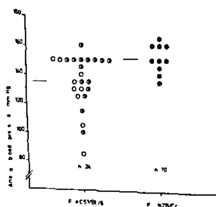
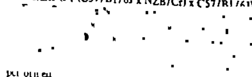


Fig 2 shows the arterial blood pressure in backcross mice either (F1 (C57/B1/6J x NZB/Cr) x C57/B1/6J)



pcr ormed

pressure in the F1 hybrids was indistinguishable from the blood pressure in the h ... Cr pa ... ous h ...

The mean values for the three groups were 154 ± 11 mm Hg (NZB/Cr) 113 ± 9 mm Hg (C57/B1/6J) and 154 ± 10 mm Hg for the (NZB/Cr x C57/B1/6J) F1 hybrids

The blood pressure in backcross mice from F1 hybrids to the two parental strains is shown in Fig 2 Backcrosses to NZB/Cr are uniformly hypertensive whereas blood pressure in backcrosses to C57/B1/6J varies from normal to high blood pressure If only one gene was responsible for the occurrence of the high blood pressure one would have expected a clear grouping among the (C57/B1/6J x NZB/Cr) x C57/B1/6J backcrosses with half the number of mice in a hypertensive group and the other half in a normotensive group However the observed uniform contribution of blood pressures in these backcross mice indicates a polygenic inheritance an assumption which is supported by the finding of a uniform mean arterial blood pressure in the F2 animals (Fig 3) which moreover is intermediate

Fig 3 shows the arterial blood pressure in F2 mice of either histocompatibility type dd (●) bb (○) or bd (◑) A higher mean value was found in the mice typing dd than in bb mice The solid line represents the mean value n = number of 3 months old male mice

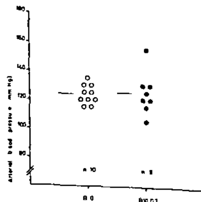


Fig 4 shows that the mean arterial blood pressure n B ...

the treatment is given early on life before the vascular disease is apparent The thymus dependency of the high blood pressure *in NZB/Cr mice* thus suggests that the high blood pressure may be an autoimmune manifestation However the influence of the incorporation of the nude alleles in the NZB/Cr genome is still only poorly understood then other explanations than the above mentioned is obviously possible

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The present study shows that also hypertension in the NZB/Cr mice is controlled by more than one gene The main influence on the blood pressure is exerted by gene(s) localized outside the major histocompatibility complex (MHC) However, the results give some evidence that loci in or closely linked to the MHC do influence the level of blood pressure It is concluded that repeated studies in larger groups of backcross generations of mice are warranted to clarify this important question

MATERIALS AND METHODS

Animals Inbred SPF NZB/Cr (H2 d) and C57/Bl/6J (H2 b) (Gl Bomholtgård Ltd Ry Denmark) were fed mouse pellets and drank tap water ad libitum Moreover B 10 (H2 b) B 10 D2 and Balb/c (H2 d) mice from Statens Seruminstitut were used

Systemic arterial blood pressures were recorded in the conscious mice In light ether anaesthesia a catheter was placed in the left carotid artery and connected to a Tybjærg Hansen capacitance pressure transducer (Simonsen & Weel Copenhagen) and a (G 14) graphic recorder (Danbridge Copenhagen) the blood pressure was subsequently recorded for one hour in the conscious semirestrained animal while it was placed in a transparent plastic tube

H 2 typing Two antisera were used in the present study A Balb/c anti C57/Bl/6J antiserum (H2 d anti H2 b) and a C57/Bl/6J anti Balb/c antiserum (H2 b anti H2 d) The antisera were obtained from mice which were immunized i p with (Balb/c x C57/Bl/6J) Fl spleen cells 8 times at weekly intervals They had cytotoxic titers of 1 256 against spleen or lymph node cells of relevant specificity The cytotoxic assay was performed as follows 0 2 ml of lymphoid cells (20×10^6/ml) was mixed with 0 2 ml of antiserum dilution (usually a 1 10 dilution in balanced salt solution = BSS) and the micture was incubated for one hour at 4° C The reaction was stopped by centrifugation the supernatant discarded and the cell pellet was resuspended in 0 5 ml of strain 2 guinea pig serum diluted 1 10 in BSS After incubation at 37° C for 45 minutes the reaction tubes were added 0 5 ml of ice cold BSS They were placed on ice until counted for presence of live cells using trypan blue due exclusion

In every assay C57/Bl/6J Balb/c and (Balb/c x C57/Bl/6J) Fl cells were used as central cells and both antisera were used being internal controls for one another By means of the present assay the antisera killed more than 90% of allogeneic cells and less than 10% of syngeneic cells More than 90% of Fl cells were killed by both antisera Thus (NZB x C57/Bl/6J) Fl F2 or backcross animals were typed as H2 d H2 b or H2 d x H2 b on the basis of whether their spleen cells were killed by only H2 b anti H2 d or H2 d anti H2 b antiserum or by both antisera All cell suspensions were counted blindly

The investigations was performed in two parts

Part I

The genetic control of the inheritance of the high blood pressure in the NZB/Cr strain of mice The systemic arterial blood pressure was measured in 15 male NZB/Cr 15 male C57/Bl/6J and in 15 male Fl (C57/Bl/6J x NZB/Cr) mice 3 months old Subsequently blood pressure was measured in 3 months old male backcross hybrids between female Fl (C57/Bl/6J x NZB/Cr) mice and either C57/Bl/6J (n = 24) or NZB/Cr (n = 10) mice

Part II

The influence of the MHC (H 2) complex on the high blood pressure The possibility of linkage of the genes coding for the high blood pressure in the NZB strain of mice to the H2 complex was investigated in backcross hybrids (Fl (C57/Bl/6J x NZB/Cr) x C57/Bl/6J) (see above) and in 53 male 3 months old F2 hybrids bred from Fl (C57/Bl/6J x NZB/Cr) parent mice The influence of genes linked to the H2 complex for the high blood pressure was further investigated by comparing the blood pressure in two congenic strains of mice which only differed at the H 2 complex i e B 10 (H2 b) and B 10 D2 (H2 d) Such male mice had their blood pressure determined when they were 3 months old

For comparison of experimental results the students t test was used When this test implies a normally distributed population a »distribution free« test which does not assume that the scores under analysis were drawn from a normally distributed population were used as well (Mann Whitney U test) The five per cent level was used as indicative of significant differences All mean values are given with one standard deviation

RESULTS

The Genetic Control of the Inheritance of the High Blood Pressure in the NZB/Cr Strain of Mice

Fig 1 shows the blood pressure in the parental NZB/Cr and parental C57/Bl/6J mice and in Fl-hybrids between the two parental strains The blood

autoimmune conditions dependent on antibodies Thus in autoimmune myasthenia gravis in rats the induction of the disease could be inhibited in rats depleted of complement by treatment with cobra venom factor (*Lennon et al* 1978) If complement deficiency is suggested as an explanation for the H 2 influence on the expression on hypertension it is important to realize that the H 2^b allele is in fact known to be connected with a low level of total complement as compared with the H 2^d allele (*Demant et al* 1973)

Finally the possibility that the NZB/Cr strain of mice which had been utilized in the present study could be different in respect to the blood pressure from the original NZB/Bl strain has not been investigated in the present study Thus further studies are warranted in order to classify this problem

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between the mean blood pressure of the two original strains (conf Fig 2 and 3)

Influence of the H-2 complex on the High Blood Pressure in the NZB/Cr strain of Mice

In Fig 2 is also included the H-2 type of the backcross hybrids to the normotensive C57/Bl/6J mice The mean value for all the mice in this group was 135 ± 19 mm Hg, as compared with 151 ± 10 mm Hg for the backcrosses to NZB/Cr When the backcrosses to C57/Bl/6J are grouped according to H-2, the heterozygous bd alleles give a mean blood pressure of 140 ± 16 mm Hg, whereas the bb homozygous mice have a mean blood pressure of 128 ± 21 mm Hg With the number of mice used in this experiment this difference is not significant ($p > 0.1$)

However, when 53 F2 hybrids were tested and grouped according to their H2 type there was a significant influence of the H2 type Fig 3 shows that mice typing dd had a higher mean blood pressure (132 + 10 mm Hg) compared with the mean value in the mice typing bb (124 ± 8 mm Hg), $p < 0.05$ and $p < 0.02$, with the Mann Whitney U test and the students' t-test, respectively The third group of F2 mice typing bd had a mean arterial blood pressure of 127 ± 11 mm Hg, which was intermediate to mean value of the other two groups

The third experimental group (Fig 4) compared the mean blood pressure in two strains of mice, congenic for the H2 complex as described in the materials and methods section Mice of the strain B 10 (H2-b) (n = 10) and mice of the strain B 10 D2 (H2-d) (n = 8) had a mean blood pressure of 124 ± 7 mm Hg and 125 ± 15 mm Hg, respectively

Here the mouse strain B 10 D2 carries the same H-2 allele as the NZB/Cr strain ($H\text{-}2^d$) Yet the mean blood pressure in the B 10 D2 mice does not differ from the mean blood pressure in B 10 which is a normotensive mouse strain carrying the same H-2 allele ($H\text{-}2^b$) as C57/Bl/6J This again shows – when compared with the above results – that the H-2 complex is not directly responsible for the development of the increased blood pressure but the H-2 complex does – provided the »high pressure gene(s)« is present – influence the expression of the increased blood pressure

DISCUSSION

The present study aimed to investigate the genetic factors influencing the inheritance and transmission of the high blood pressure in the NZB/Cr strain of mice (*Svendsen* 1977) Because evidence has been provided that the renal autoimmune disease in the F1 (NZB X NZW) mice are controlled by two dominant genes, one of which is linked to the H-2 complex (*Knight & Adams* 1976) it was investigated if the genes coding for the high blood pressure in the NZB/Cr mice might be linked to this complex The earlier mentioned point that thymus is essential for the development of high blood pressure in the NZB/Cr mice would seem to indicate that some kind of T-cell function is involved The present investigation shows that the spontaneous hypertension in the NZB/Cr strain of mice is controlled by more than one – but probably few autosomal dominant genes Previously, we found a high blood pressure in the BALB/c/A and the NZB/Cr strain of mice both of which carry the H2-d allele of the major histocompatibility complex (*Svendsen* 1977) However, the results obtained in the present study using mice congenic for the H2 complex (H2-d and H2-b, respectively) do not support this assumption because the same mean blood pressure within the normal range of C57/Bl/6J was found in both strains, irrespectively of the H2 type

Thus, we must conclude that the gene(s) which determine the development of the hypertension is located outside the major histocompatibility complex We have not had the possibility to test for linkage to other well characterised gene markers in the mouse An obvious candidate here would be loci controlling allotypes of mouse immunoglobulins (heavy and light chains)

Although it is found that genes, linked to the H-2 complex are not themselves responsible for the development of the high blood pressure in the NZB/Cr mice the experiments with backcross and F2-hybrids gave a weak evidence, that the hypertension is influenced by genes linked to the MHC In brief, these genes are known to influence T effector functions T helper functions and the level of certain complement components (*Svejgaard et al* 1975 *Klein* 1975) Repeated studies with larger groups of backcross generations of mice are highly warranted

Previously, we have shown that in another hypertensive system (renal hypertension in rats) (*Svendsen* 1973) thoracic duct lymphocytes from hypertensive rats when transferred into normal syngeneic rats caused a »secondary response« in the cellular reaction against damaged arteriolar walls following later injections of angiotensin II These findings thus suggest that T helper cells are involved in at least some kinds of hypertension

The fact that in the spontaneous hypertension in the NZB/Cr mice the hypertension could be a manifestation of an autoimmune disease might, however, indicate that the complement system could be involved This has been shown for several

autoimmune conditions dependent on antibodies Thus in autoimmune myasthenia gravis in rats the induction of the disease could be inhibited in rats depleted of complement by treatment with cobra venom factor (*Lennon et al* 1978) If complement deficiency is suggested as an explanation for the H 2 influence on the expression on hypertension it is important to realize that the H 2^b allele is in fact known to be connected with a low level of total complement as compared with the H 2^d allele (*Demant et al* 1973)

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MITOGENIC STIMULATION OF HUMAN LYMPHOCYTE SUBPOPULATIONS BY *LATHYRUS ODORATUS* LECTIN

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Kolberg J & Michaelsen T E Mitogenic stimulation of human lymphocyte subpopulations by *Lathyrus odoratus* lectin Acta path microbiol scand Sect C *87* 275-279 1979

Investigation was made of the mitogenic activation by *Lathyrus odoratus* (LATH) lectin and phytohaemagglutinin (PHA) of human T-enriched and non T lymphocytes isolated by nylon wool filtration and sheep erythrocyte rosette depletion respectively LATH stimulated only weakly the T enriched and non T fractions as compared to PHA which strongly stimulated the T-enriched population and weakly stimulated the non T cells Removal of phagocytic cells from the unfractionated lymphocytes caused only a slight reduction in the response to LATH The responses of mixtures of T enriched and non T lymphocytes were similar to that of unfractionated lymphocytes The LATH lectin seems therefore to be neither a pure T nor a pure non T cell mitogen since both populations must be present for optimal response When α methyl D mannoside was added to unfractionated lymphocytes together with LATH there was a strong inhibition of thymidine incorporation D-glucose and N acetyl D glucosamine diminished the stimulation effect to a lesser degree

Key words Lectin mitogen lymphocytes *in vitro*

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Lectins have been found in extracts of many different plants mostly legumes as well as in microorganisms and invertebrates (9) Several lec

[illegible]

... the seeds of *Lathyrus odoratus* (LATH) belonging to the *Leguminosae* family has a strong mitogen effect on human peripheral blood lymphocytes (12) The mitogenic response to LATH in terms of ^{3}H thymidine incorporation was approximately the same as for phytohaemagglutinin (PHA) but the weight concentration of LATH giving max... [illegible]

... tions of human lymphocytes and the inhibitory effect of different carbohydrates on the mitogenic activity of LATH

MATERIALS AND METHODS

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Lymphocytes were isolated from heparinized (without preservative) blood according to the method of *Bøyum* (4) The cells were washed three times in Hanks balanced salt solution (HBSS) These cells will be referred to as unfractionated lymphocytes

T enriched lymphocytes were prepared by filtration through nylon wool (Fenwal Lab Morten Grove Ill) (21)

T cell depletion The unfractionated lymphocytes were adjusted to 20×10^6 cells/ml in HBSS with 20 per cent pooled heat inactivated human AB serum which had previously been absorbed with sheep erythrocytes (SRBC) This lymphocyte suspension was mixed with an equal volume of 5 *per cent* SRBC in HBSS and centrifuged at $250 \times g$ After incubation for 1 - 1 5 *h* at 4° C the rosette forming lymphocytes (T-cells) were separated from the other cells (non T cells) by centrifugation on Isopaque Ficoll (4)

Depletion of phagocytic cells was performed by iron treatment (Technicon Product No T01 0507) (18)

TABLE 2 *Incorporation of ³H thymidine into Lymphocytes Stimulated with PHA or LATH*

Expt. no	Mitogen	Unfractionated cells	T-enriched cells		Non T cells	
1	PHA	199 ± 19[a]	210 ± 20[a]	(106)[b]	32 8 ± 2 6[a]	(16)[b]
	LATH	199 ± 9 5	65 5 ± 5 5	(33)	10 5 ± 2 6	(5 2)
2	PHA	142 ± 3 0	146 ± 36	(103)	24 7 ± 3 2	(17)
	LATH	125 ± 10	18 0	(4)	13 2 ± 1 4	(11)
3	PHA	175 ± 12	215 ± 23	(123)	35 8 ± 4 0	(20)
	LATH	86 7 ± 23	27 7 ± 4 9	(32)	17 3 ± 2 9	(20)
4	PHA	194 ± 11	82 8 ± 16	(43)	32 0 ± 1 7	(16)
	LATH	139 ± 17	15 8 ± 4 2	(11)	27 9 ± 3 9	(20)

[a] (cpm/culture ± SD) × 10³

[b] percentage of incorporation compared with that of unfractionated cells

TABLE 3 *LATH Stimulation of Unfractionated Lymphocytes before and after Depletion of Phagocytic Cells*

Expt no	Unfractionated lymphocytes	Iron treated lymphocytes	
1	96 3[a]	92 2[a]	(96)[b]
2	133	102	(77)
3	163	109	(67)

cpm/culture × 10³

percentage of incorporation compared with that of untreated cells

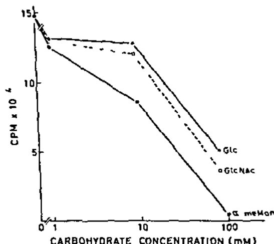


Fig 3 Effect of carbohydrates on LATH stimulation of thymidine incorporation into 3-day cultures of unfractionated lymphocytes Glc D glucose GlcNAc N acetyl D glucosamine α meMan α methyl D mannoside

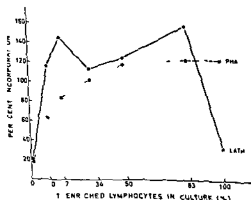


Fig 2 Thymidine incorporation into 5-day cultures of mixtures of T-enriched and non T lymphocytes exposed to PHA or LATH lectin The results are expressed as percentage of incorporation compared with that of unfractionated lymphocytes

Mitogenic responses of mixtures of T enriched and non T cells In order to test whether or not the T and the non T lymphocytes co-operate in their responses to LATH the mitogenic responses of mixtures of T-enriched and non T cells were measured Synergy between T and non T cells is demonstrated by the fact that thymidine incorporation by the cell mixtures is higher than the sum of T-enriched and non T cells cultured separately (*Fig* 2) PHA stimulated strongly the T-enriched cell fraction The PHA response of non T lymphocytes mixed with various numbers of T-enriched cells

Identification of T lymphocytes T cells were identified on the basis of rosette formation with SRBC Briefly, equal volums of lymphocytes (5×10^6 cells/ml) and 0 5 per cent SRBC were mixed and centrifuged at $250 \times g$ The cell mixture was incubated overnight at 4° C All lymphocytes binding three or more SRBC were considered positive

Identification of Ig-bearing cells FITC-labelled sheep anti human $F(ab')_2$ was used to identify membrane Ig-bearing cells by immunofluorescence microscopy (7)

Identification of monocytes was carried out by peroxidase staining (11) on preparations made by cytocentrifuge (Shandon Scientific Co Ltd , London)

Culture and assay procedure The lymphocytes were cultivated in Medium 199 supplemented with 10 per cent pooled, heat-inactivated, human AB serum, penicillin and streptomycin Cultures were set up in flat-bottomed Nunclon microplates (Roskilde, Denmark) with $0\ 15 \times 10^6$ cells in 150 µl medium per well, and incubated at 37° C in a humidified atmosphere of CO_2 in air The mitogens were added in 10 µl saline at zero time The concentration used was 0 6 µg/ml of PHA or 40 µg of LATH (12)

Twenty-four h before harvesting 0 7 µCi of ^{3}H-thymidine (The Radiochemical Centre, Amersham England) was added The cells were harvested with distilled water in an automatic harvesting machine (Skatron Lierbyen Norway) The glass fibre filters were put directly into counting vials and incubated for about 20 h in 5 ml Dilusolve scintillation fluid (Packard Instrument Ltd) During this period the counting efficiency increased to a stable level The radioactivity was measured in Packard spectrometer, model A 2425 operated at room temperature The experiments were performed in triplicate or quadruplicate

RESULTS

Mitogenic stimulation of unfractionated T-enriched and non-T cell fractions Human peripheral blood lymphocytes were separated into T-enriched and non-T cell fractions by nylon wool filtration and by SRBC rosette depletion, respectively In some experiments the Ficoll-Isopaque isolated cells (unfractionated lymphocytes) were treated with iron to remove phagocytic cells These preparations were characterized by immunofluorescence staining for membrane-bound immunoglobulin, rosette formation activity with SRBC and staining with peroxidase (Table 1)

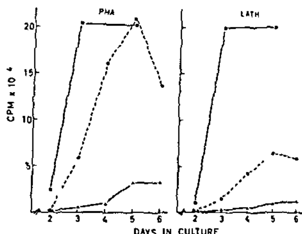


Fig 1 Thymidine incorporation into cultures exposed to PHA or LATH lectin ■——■ unfractionated lymphocytes ●------● T-enriched cells and ▲——▲ non-T cells

Unfractionated, T-enriched and non-T lymphocytes were incubated with the LATH lectin or PHA for varying periods of time (Fig 1) This representative experiment indicates that these lymphocyte preparations responded maximally to both mitogens after approximately five days of incubation and thus that period was chosen for the further studies PHA was a much stronger T cell mitogen than LATH, the PHA response being 3–8 times that of LATH (Table 2) In several experiments unfractionated and T-enriched lymphocyte fractions had similar capacities for stimulation by PHA The non-T cells responded weakly to both mitogens Removal of phagocytic cells from unfractionated lymphocytes caused only a slight reduction in the LATH response (Table 3)

TABLE 1 *Characteristics of Human Blood Lymphocyte Preparations*[a]

	SRCB rosette forming cells	Staining with FITC-labelled anti-F(ab')₂	Staining with peroxidase
Unfractionated lymphocytes	60 ± 8 2	17 ± 7 3	24 ± 3 5
Iron treated lymphocytes	64 ± 16	7 ± 4 2	2 3 ± 1 0
T-enriched lymphocytes	83 ± 12	1 8 ± 1 3	<0 5
Non T lymphocytes	1 2 ± 2 0	72 ± 4 9	48 ± 6 1

[a] Values shown are mean percentages ± SD

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cpm/culture × 10³

percentage of incorporation compared with that of untreated cells

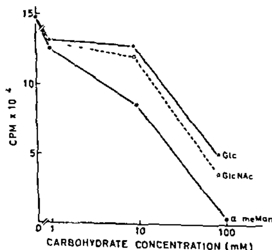


Fig 3 Effec...

D glucosamine α meMan α methyl D mannoside

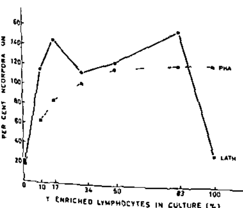


Fig 2 Thymidine incorporation into 5-day cultures of mixtures of T-enriched and non T lymphocytes exposed to PHA or LATH lectin The results are expressed as percentage of incorporation compared with that of unfractionated lymphocytes

Mitogenic responses of mixtures of T-enriched and non-T cells In order to test whether or not the T and the non T lymphocytes co-operate in their responses to LATH the mitogenic responses of mixtures of T-enriched and non T cells were measured Synergy between T and non T cells is demonstrated by the fact that thymidine incorporation by the cell mixtures is higher than the sum of T-enriched and non T cells cultured separately (Fig 2) PHA stimulated strongly the T-enriched cell fraction The PHA response of non T lymphocytes mixed with various numbers of T-enriched cells

seemed to be not proportional to the number of T-enriched cells A plateau of maximum stimulation was observed at the highest T-enriched cell concentrations (Fig 2)

Inhibition of mitogenic activity with monosaccharides When α-methyl-D mannoside was added to unfractionated lymphocyte cultures together with the LATH lectin for three days, there was strong inhibition of the incorporation of labelled thymidine (Fig 3) D-glucose and N acetyl-D-glucosamine diminished the stimulating effect to a lesser degree D-galactose, N acetyl-D-galactosamine and L-fucose had some inhibitory effect (not shown), but not in all experiments

DISCUSSION

In this study we have compared the mitogenic stimulation of the *Lathyrus odoratus* (LATH) lectin on human T-cell enriched and T-cell depleted (non-T) preparations with that of PHA LATH gave a weak stimulation of both lymphocyte preparations, while PHA strongly stimulated the T-cell enriched fraction and weakly stimulated the non-T cell fraction The strongly reduced LATH response of the T-enriched fraction is probably not due to removal of monocytes by the nylon wool filtration since depletion of phagocytic cells from unfractionated lymphocytes resulted only in a slightly reduced response Our findings that purified T cells are strongly stimulated by PHA are in accordance with those of other groups (3, 15, 16, 17) However there are many conflicting reports with regard to the response of non T lymphocytes to PHA It has been demonstrated that human non-T cells isolated from immunoabsorbent columns incorporate thymidine in the presence of PHA (5, 14) On the other hand, non-responsiveness of cells isolated by this technique has also been reported (3) In accordance with the findings of other groups (2, 16), we found a weak PHA response of non-T cells isolated by depletion of sheep erythrocyte rosetting T cells Even complete non-responsiveness of cells isolated by this depletion technique has been reported (3, 10, 20) These differences in the stimulation capacity of PHA towards non-T lymphocytes can probably be explained by differences in the techniques used for isolation of the cells and/or differences in the purity of the cell populations obtained

It has been claimed recently there are subpopulations of lymphocytes which have neither T cell nor B cell markers (for review see 6) This makes the whole field even more complex, since these cells have not been used in extensive mitogenic studies Other cells such as monocytes might also interact with the mitogenic response However, as already mentioned, this does not seem to be the case for LATH The non-T cells isolated by depletion of rosetting T cells can be heavily contaminated with monocytes, as shown here and by others (1, 8, 18)

There is also evidence that lymphocyte subpopulations co-operate in their response to mitogens such as PHA and pokeweed mitogen (PWM) (3, 8, 15, 20) Using LATH, we also observed a striking synergy Thus LATH seems to be neither a pure T nor a non-T cell mitogen, but requires both populations for optimal response The mechanism behind the mitogenic stimulation of lymphocytes is not clearly understood therefore, a high mitogenic response characteristic for mitogens such as LATH and PHA (15, 20) as compared to mitogens which give a low response like PWM (15, 20) might reflect different mechanisms of activation

With regard to the ligand specificity of LATH on lymphocytes, the mitogenic activation was strongly inhibited by α-methyl-D mannoside and to a lesser degree by D-glucose and N-acetyl D glucosamine Lectins are generally not specific for one single sugar only, although the degree of specificity varies widely The lectins have been classified into nine groups according to their main sugar affinities (13) The results with the LATH lectin indicate that it belongs to group 8 in this classification, together with lectins such as *Canavalia ensiformis* (Con A) *Pisum sativum* and *Lens culinaris* It has also been reported that the mitogenic effect of these lectins is inhibited by α methyl D mannoside (19, 22)

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CYTOLOGICAL EVENTS IN ALLO-STIMULATED LYMPHOCYTES TRIGGERED BY EXPOSURE TO STIMULATORY ALLOANTIGENS

III Changes in the Areal Density of Cytoplasmic Vacuoles containing Endocytized Thorotrast®

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Brix Poulsen P & Nielsen L H Cytological events in allostimulated lymphocytes triggered by exposure to stimulatory alloantigens III Changes in the areal density of cytoplasmic vacuoles containing endocytized Thorotrast® Acta path microbiol scand Sect. C 87 281–286 1979

H 2b lymphocytes were sensitized against H 2d alloantigens by a lymphocyte culture reaction (MLR) and incubated with H 2d mastocytoma cells Thirty minutes before incubation with the mastocytoma cells Thorotrast® was added to the lymphocyte culture medium The interaction between lymphoid cells and mastocytoma cells was stopped by fixation with glutaraldehyde The areal densities of the cytoplasmic vacuoles were examined by electron microscopy Two populations of lymphocytes were observed small lymphocytes with heterochromatic nuclei and larger lymphocytes (lymphoblasts) with euchromatic nuclei Only the lymphoblasts showed changes following interaction with target cells The areal density of Thorotrast® containing vacuoles in sensitized lymphoblasts increased during the three hour observation period but the percentage of vacuoles containing Thorotrast® in the total area of cytoplasmic vacuoles decreased in the first thirty minutes of interaction with target cells This observation may indicate greater formation of vacuoles from the Golgi apparatus compared with the formation of vacuoles from the cytoplasmic membrane at the onset of interaction with target cells In the multivesicular bodies (MVB) the Thorotrast® was first observed inside the small vesicles and later on it was also observed in the matrix of the MVB After three hours of interaction Thorotrast® was observed laying »free« in the matrix of phagosomes along with myelin figures

Key words Lymphoid cells lysis vacuoles Thorotrast®

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It has been demonstrated that phytohaemagglutinin (PHA) stimulation of lymphocytes induces increased uptake of a wide range of molec[illegible]
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from culture medium (*Biberfeld* 1971 and *Hirschhorn et al* 1968)

An increase in synthesis of membran associated lipid has been shown to occur rapidly in lymphocytes after stimulation perhaps also reflecting the increase in endocytosis (*Fischer & Mueller* 1968)

Protein molecules are taken up by cells by micropinocytosis and degraded in the lysosomal system The vacuolar system appears to be engaged

in the uptake transport and digestion of endocytized material (*de Duve & Wattiaux* 1966)

Biberfeld (1971) using human peripheral blood lymphocytes transformed by PHA indicated diffe rent morphological stages of the vacuolar system in a sequential transformation process from endocytic vesicles to dense bodies through different types of multivesicular bodies (MVB) Other observations indicate that MVB also may arise from the Golgi apparatus (*Ericsson* 1964 *Friend* 1969 and *Holtz man et al* 1967)

Some of the prerequisites for the formation of vacuoles in mixed lymphocyte reaction (MLR) stimulated lymphocytes during interaction with target cells – a readiness of the granulated endoplasmic reticulum and the Golgi apparatus – was discussed in a previous study (*Brix Poulsen* 1979) The study suggested two »generations« of the Golgi apparatus in the lymphoblasts The first »generation« increased from the onset of interac tion but after twenty minutes of interaction this »generation« of the Golgi apparatus decreased The second »generation« increased after one hour of interaction The maximum concentration of the granulated endoplasmic reticulum in the lymphocy tes was found at the onset of interaction with target cells

In another study (*Brix Poulsen & Nielsen* 1979) two main periods of vacuole formation in MLR stimulated lymphocytes during interaction with target cells were demonstrated – one from the onset to thirty minutes and one from the second to the third hour of interaction

The aim of the present study using Thorotrast® in culture medium was to examine the origin of the vacuoles – whether the formation is from the Golgi apparatus or from the cell surface – during the two main periods of vacuole formation in the lymphocy tes during interaction with target cells

MATERIAL AND METHODS

Animals Mice of inbred strains DBA/2J (H 2^d) and C57Bl/6J (H 2^b) were obtained from Gl Bomholtgård Laven Jutland F_1 *hybrids of C57Bl/6J × DBA/2J* were bred at the Institute of Medical Anatomy Copenhagen

Cells Effector cells C57Bl 6J lymphocytes from spleen and lymph nodes *Stimulator cells* F_1[C57Bl/6J × DBA/2J] lymphocyte from spleen and lymph nodes *Target cells* The tumour P 815 × 2 mastocytoma of the H 2^d origin was kindly donated by Dr *Bent Rubin* Statens Seruminstitut Copenhagen

Cell preparation Spleen and lymph nodes were obtained aseptically and homogenized by grinding through a Monodur® P A extra stark 400 The cells were washed once in 4° C phosphate buffered saline pH 7 2 (PBS 420 Gibco) and the cell debris was allowed to settle The cells were then spun down and resuspended in 156 6 mM NH_4Cl in distilled water at 4° C for 10 minutes which disrupted the erythrocytes After three washes the cells were resuspended in culture media and counted

Cultivation medium 500 ml RMPI 1640 25mM with Hepes buffer (240 S1 Gibco) containing 25 ml (5%) foetal calf serum (629 Gibco) 5 ml (1%) L glutamine 200 mM (503 Gibco) 125 000 i u Penicillin (Novo) 0 05 g Streptomycinsulfat (Novo) and 5 × 10^{-2} mM 2 mercaptoethanol

Cultivation medium containing Thorotrast® The same as »cultivation medium« plus 0 025 ml 25% stabilized colloidal thorium dioxid (Thorotrast® No 504 A Fellow test agar Detroit) per ml medium

Stimulation of lymphocytes 30 × 10^6 stimulator cells and 30 × 10^6 effector cells were mixed in Falcon tissue culture flask No 3018 with 20 ml cultivation medium and stored for 4 days at 37° C in an atmosphere containing 5% CO_2

Cytolysis fixation and embedding Day 4 after initiating the stimulation the lymphocyte suspensions were counted and placed in the cultivation medium containing Thorotrast® After thirty minutes half of the lymphocytes were mixed with mastocytoma cells in a 10 1 lymphocyte mastocytoma cell ratio adjusted to 30 × 10^6 cells per ml cultivation medium and shaked The other half of the lymphocytes stayed in the cultivation medium with Thorotrast® and without target cells and became *the control cells*

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Electron microscopy Silver to gray thin sections were cut on a LJB Ultrotome stained with uranyl acetate and lead citrate the cells were examined and photographed using a Philips EM 300 electron microscope at an accelerating voltage of 60 kV

Quantitation and statistics The cytoplasmic areas and vacuole areas were measured and calculated stereologi cally with a Leitz A S M image system

The Wilcoxon non parametric test was used for statistical analysis of the cytological changes Number of cells counted In each observation period 40 lymphocytes were measured along with equal numbers of control cells

RESULTS

The cell population after mixed lymphocyte reaction (MLR) stimulation consisted of two types of lymphoid cells a small type with heterochromatic nucleus and a larger type with euchromatic nucleus Only the latter exhibited changes following interaction with target cells This cell resembles a blast cell and the quantitation was performed on this cell type only

The areal density of cytoplasmic vacuoles containing Thorotrast® The blast cells showed an increase in the areal density of vacuoles containing Thorotrast® from 0 28% to 0 86% within the first 30 minutes of interaction with target cells (Table 1) In the next hour there was no significant increase in the density From two to three hours of interaction the density of vacuoles with Thorotrast® increased from 1 18% to 1 63%

The control cells showed no significant change in

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Time of interaction (hours)	The area of vacuoles containing T in per cent of cytoplasmic area (%)	Probability (P)	The area of vacuoles containing T in per cent of the total vacuoles area (%)
0	0 28		17 2
½	0 86	p<0 05	8 3
1	0 94	p<0 20	15 0
2	1 18	p<0 20	24 3
3	1 63	p<0 025	27 5



Fig 1 Thorotrast® inside small vesicles of two multivesicular bodies in lymphocyte after 30 min of interaction with target cells 33000 ×

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some of the small vesicles of multivesicular bodies (MVB) whereas the matrix of MVB did not contain Thorotrast® (Fig 1) After one hour of interaction with target cells Thorotrast® was located in the matrix as well as inside the small vesicles of MVB (Fig 2 and 3) After two hours of interaction Thorotrast® was also observed in small vesicles (<0 2μ) inside phagosomes containing myelin figures (Fig 4) After three hours of interaction Thorotrast® was also seen distributed in the matrix of phagosomes along with myelin figures (Fig 5 and 6)

in the uptake, transport and digestion of endocytized material (*de Duve & Wattiaux* 1966)

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The localization of Thorotrast® inside the vacuoles During the first 30 minutes of interaction with target cells the Thorotrast® was observed inside some of the small vesicles of multivesicular bodies (MVB) [illegible]

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Fig 4 Thorotrast® inside two small vesicles of a phagosome with a myelinfigure in lymphocyte after two hours of interaction with target cells 50000 ×

cells were in a state of readiness for formation of cytoplasmic vacuoles Thus the highest concentra tion of endoplasmic reticulum was found in the relatively small blast cells from the beginning of interaction with target cells The Golgi stacks also showed a readiness because the number of stacks per μ^2 of cytoplasma is only one third of the initial number after twenty minutes of interaction

The formation of vacuoles and lysosomes in the blast cells during the interaction with target cells has been quantified (*Brix Poulsen & Nielsen* 1979) Two main periods of vacuole formation were found – one from the onset to thirty minutes and one from the second to the third hour of interaction The formation of lysosomes in the blast cells was greatest from two to three hours of interaction but acid phosphatase was observed in multivesicular bodies (MVB) after twenty minutes

The present experiment indicates that in the first thirty minutes vacuoles are formed by the Golgi apparatus as the percentage of vacuoles containing Thorotrast® in the total vacuole area decrease in the first thirty minutes This is in agreement with the readiness state of the Golgi apparatus and the endoplasmic reticulum Later the increase *in the* percentage of Thorotrast® containing vacuoles in the total vacuole area indicates that the building materials had to come from the environments This is in agreement with the observation of *Hayden et al* (1970) who found a doubling of the rate of incorporating glucosamine into membrane glycoproteins within 3 hours following add tion of PHA to human peripheral blood lymphocytes

An increase in uptake may explain the increase in lysosomes to break down macromolecules to different building units (*Hirschhorn et al* 1967 *Brix Poulsen et al* 1975 and *Brix Poulsen & Gutler* 1975 and *Brix Poulsen & Nielsen* 1979)

In PHA-stimulated lymphocytes there is an increase in the biosynthesis and turnover of membrane constituents (*Hayden et al* 1970) It has also been shown that membrane immunoglobulins and histocompatibility antigens turn-over rapidly (*Vitetta et al* 1972) After interaction with target cells there is a redistribution of surface immunoglo-





Fig 5 and 6 Thorotrast® distributed in the matrix of phagosomes along with myelinfigures Fig 5 45300 × Fig 6 43920 ×

DISCUSSION

In a previous study (*Brix Poulsen* 1979), it has been shown that the mixed lymphocyte reaction (MLR) stimulated blast cells increased their diameter during the first three hours of interaction with the stimulating alloantigens In another study (*Cohnen* 1975) an increase in the cell diameter of lymphocytes after three to five days of phytohaemagglutinin (PHA) stimulation was found

A prerequisite for the blast cell to increase its diameter is sufficient amount of building materials for the cell membrane synthesis These building materials may solely come from the cell itself without increased uptake into the cell, but a larger increase in cell membrane area requires a sufficient uptake from the environments

In an earlier study (*Brix Poulsen* 1979) it was observed that following MLR-stimulation, the blast

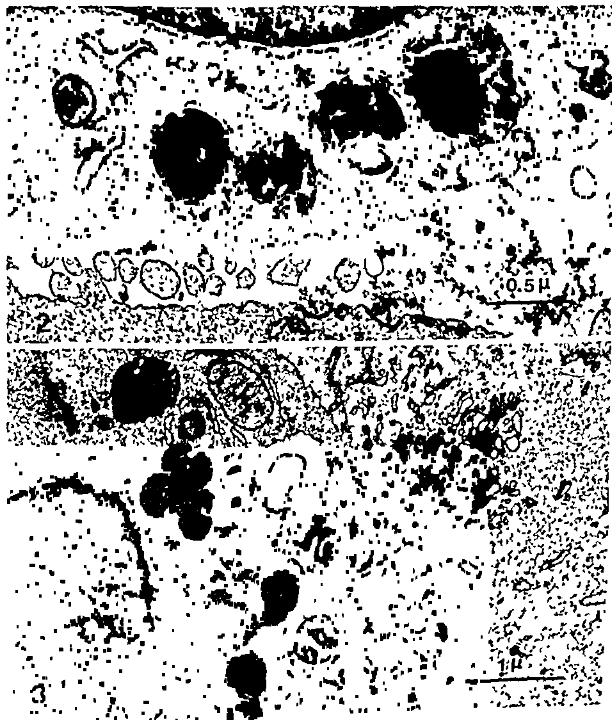


Fig 2 and 3 Thorotrast® located as well in the matrix as inside the small vesicles of multivesicular bodies in lymphocytes after one hour of interaction with target cells Fig 2 39560 × Fig 3 24380 ×

Acta path microbiol scand Sect C 87 287–292 1979

DO BURSA-DERIVED T CELLS EXIST?

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Lassila O Do bursa-derived T cells exist? Acta path microbiol scand Sect C 87 287–292 1979

In order to study the bursal origin of T cells 18-d chromosomally marked bursa cells were transplanted into 18-d chick embryos treated with cyclophosphamide (CY) Transfer of bursa cells restored not only the humoral immune functions but also the mitogenic responses of peripheral blood lymphocytes against phytohaemagglutinin and concanavalin A (Con A) Transplanted bursa cells proliferated primarily in the recipients bursa Only 8 out of 209 Con A responsive spleen cells were of donor origin but no donor-derived cells were observed in the thymus These findings suggest that cells in the 18-d embryonic bursa are already committed to the B cell lineage and are not capable of migrating into thymus and of developing into mature T cells

Key words Bursa lymphoid differentiation T cells

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The primary lymphoid organs of the chicken thymus and bursa of Fabricius are colonized by blood borne stem cells Recently evidence has been presented that these stem cells originate primarily in the early embryo itself (9 10) and not in the yolk sac, as has generally been supposed (13 14) Stem cells immigrating to the thymic or bursal primordia differentiate to either T or B lymphocytes under the influence of the respective epithelium During maturation the cells become restricted to T or B cell lineage and loose their stem cell characteristics However it is not known at what stage of development the cells become committed to the T or B cell maturation

At present there is no direct evidence to suggest that bursal cells are capable of colonizing the thymus On the basis of results obtained from quail [illegible]

results in increased thymus weight and concanavalin A (Con A) responses as compared to the untransplanted controls (4 5) Likewise early bursectomy at 70 hours of incubation results in an incomplete development of the thymic cortex (6) In addition *Droege* (2) and *Zucker et al* (26) have proposed the existence of a bursa-dependent subpopulation in the thymus of young chickens These findings are supported by the demonstration of bursa marker positive cells without any B cell function in the thymus of the chicken until two weeks of age (7) It has also been suggested that thymic factors may induce *in vitro* T cell characteristics on the bursa cells (1 17)

The potential existence of bursa-derived T cells has recently been studied by *Weber & Alexander* (22) They transplanted chromosomally marked bursa cells taken at 13 days of incubation into 13-day-old irradiated embryonic recipients and used chromosome analysis of mitogen stimulated cells at 5–12 wks of age to study the donor cell contribution to the T cell system of the recipients The results obtained did not reveal any evidence for the existence of bursa-derived T cells Comparable results were also obtained when 19-day embryonic bursa cells were transferred into 5-day-old recipients treated with cyclophosphamide (23) This problem has also been investigated in the present

bulins and histocompatibility antigens on the lymphocyte surface (*Perkins et al* 1972 and *Mc Intyre & Karnovsky* 1973)

During the first minutes of interaction with target cells uptake of vacuole membranes into cytoplasma membrane might provide structures to the outer cell surface from the inner surface of vacuoles Such structures may be necessary for the recognition of the target cell in the recognition phase of cytolysis as described by *Golstein & Smith* (1977) This recognition phase is finished very quickly because an electrolyte permeable lesion is produced in the target cell membrane within minutes following contact with the cytolytic T lymphocytes (*Martz* 1976) The vacuoles without Thorotrast® formed at the onset may then be responsible for providing structures for the recognition of the target cell The lysosomes formed later (*Brix Poulsen* 1979) may be engaged in breaking down the content of the Thorotrast® loaded vacuoles

If all vacuoles were formed from the cell surface one would expect an unchanged percentage of vacuoles containing Thorotrast® *in the total number* of vacuoles Therefore the decrease in vacules containing Thorotrast® at the onset indicates a formation of vacuoles from the Golgi apparatus because there is no Thorotrast® in the Golgi apparatus

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TABLE 2 *Transplantation of Embryonic Bursa Cells into CY Treated Chick Embryos Effect on Anti Brucella and Anti HGG Antibodies*

Antibody	Normal controls (N = 5)	CY treated chickens (N = 7)	Bursa cell transplanted chickens (N = 8)
IgM anti *Brucella*			
0	0 127 ± 0 038	0 135 ± 0 040	0 148 ± 0 048
1°	0 650 ± 0 285[a]	0 115 ± 0 053	0 427 ± 0 189[b]
2°	0 756 ± 0 103[a]	0 127 ± 0 044	0 589 ± 0 275[a]
IgG-anti *Brucella*			
0	0 063 ± 0 010	0 066 ± 0 012	0 063 ± 0 019
1°	0 250 ± 0 133[b]	0 076 ± 0 018	0 166 ± 0 059[c]
2°	0 498 ± 0 189[a]	0 065 ± 0 030	0 365 ± 0 302[b]
IgM anti HGG			
0	0 197 ± 0 044	0 170 ± 0 031	0 164 ± 0 047
1°	1 115 ± 0 089[a]	0 176 ± 0 059	0 883 ± 0 250[a]
2°	1 131 ± 0 199[a]	0 198 ± 0 072	0 924 ± 0 187[a]
IgG anti HGG			
0	0 116 ± 0 052	0 110 ± 0 021	0 106 ± 0 047
1°	0 947 ± 0 077[a]	0 113 ± 0 043	0 806 ± 0 103[a]
2°	1 009 ± 0 145[a]	0 131 ± 0 048	0 795 ± 0 250[a]

Measured as a proportion of standard serum by ELISA

[a] P<0 001
[b] P<0 01
[c] P<0 05
} as compared to CY treated chickens

TABLE 3 *Transplantation of 5 × 10⁶ 18 Day Embryonic Bursa Cells into CY Treated Embryos[a] Effect on Mitogenic Responses of Peripheral Blood Lymphocytes*

Age of chickens (wks)	Mitogen	Normal controls (N – 8)	CY treated chickens (N – 8)	Bursa cell transplanted[b] chickens (N = 6)
5	–	227 ± 73	195 ± 53	133 ± 112
	PHA	59936 ± 15312[d]	30655 ± 8937	49311 ± 15157[d]
	Con A	32737 ± 9953[d]	12386 ± 8712	30764 ± 10503[d]
7	–	307 ± 105	295 ± 151	235 ± 75
	PHA	50844 ± 11355[d]	28878 ± 5307	47493 ± 16435[d]
	Con A	40426 ± 8173[d]	20113 ± 9785	43166 ± 12305[d]
11	–	151 ± 32	236 ± 78	N D
	PHA	47603 ± 16345[d]	25194 ± 7942	N D
	Con A	46253 ± 7003[d]	18693 ± 11339	N D

[a] 1 50 mg/embryo/day on the days 15 16 and 17 days of incubation
[b] On day 18 of incubation
[c] Mean ± SD are given
[d] P < 0 05 as compared to CY treated controls

study over the past two years The observations made by *Weber* were confirmed and extended by using CY-treated chicken embryos (3) as cell recipients, as well as by applying functional parameters to measure T cell mediated immunity In this model, the bursa is depleted from stem cells more completely than after X-irradiation, and the potential donor cell contribution might be more easily detectable

MATERIAL AND METHODS

Chickens White Leghorn line P chickens from our own colonies were used *This line is homozygous for the* major histocompatibility locus *genotype* B^2B^2

CY treatment CY treatment was carried out as described earlier (3) CY was injected intravenously on three consecutive days, *beginning on day 15 of incubation* The daily dose was 1 50 mg/0 1 ml/embryo

Bursa cells 18-day-old histocompatible male embryos were used as donors for bursa cells Bursas were dissected and cut with scissors into small pieces in medium TC 199 (Orion, Helsinki, Finland) The medium was supplemented with antibiotics (penicillin 100 U/ml, streptomycin 100 μg/ml) and heparin (5 U/ml) The bursal tissue was homogenized in Tenbroeck-type tissue grinders To remove the cell clusters and debris, the cell suspensions were allowed to settle for 10 minutes, after which the supernatant was collected and the procedure repeated The cells in the supernatant were washed twice with the medium The cells were adjusted to a concentration of 5×10^7 cells/ml and 0 1 ml of this bursa cell suspension was injected intravenously on day 18 of incubation

[illegible]

injected intraperitoneally at the age of 4 weeks The birds were bled 7 days after the first stimulation and 5 days after the second stimulation IgM and IgG antibodies against HGG and *Brucella* were quantified by enzyme linked immunosorbent assay (ELISA) as a modification of a RIA described previously (21)

Lymphocyte response to mitogens The responses of peripheral blood lymphocytes against phytohaemagglutinin (PHA) and Con A were determined by a whole blood microassay (11) Cultures were performed in triplicate

Chromosome analysis At the age of six weeks the chickens were weighed, killed with ether and autopsied The bursa and spleen of the chickens were weighed Only female birds were processed further Cell suspensions from thymus, bursa and spleen were prepared for chromosome analysis as described above From bursa cells, direct chromosome preparations were made by incubating bursa cells with demecolchicine (Colcemide Fluka, Switzerland) for three hours (15) In order to study T cells selectively, chromosome preparations from thymus and spleen cells were made after stimulation with Con A (18) The cells were cultured for 48 hours in serum free medium (RPMI 1640 Grand Island Biological Company, Grand Island New York) with optimal dose of Con A (1 3 μg/ml, Pharmacia Fine Chemicals Uppsala, Sweden) To block the cells into metaphases Colcemide® was added for the last three hours of incubation Control cultures were performed in the same way but without mitogen stimulation Chromosomes were prepared, using a hypotonic KCl (0 075 M) solution fixed three times with glacial acetic acid/methanol (1 3) Chromosome preparations were stained with 5% Giemsa (Fluka, Switzerland)

Statistics Student's *t* test was employed in the comparison of means

RESULTS

The capacity to restore the humoral immune functions of the CY-treated chicken embryos by

TABLE 1 *Transplantation of* 5×10^6 *18-Day Embryonic Bursa Cells into CY-Treated Embryos*[a] *Effect on Body and Organ Weights*[b]

CY treatment	Cell transfer[c]	No of chickens	Body weight (g)	Bursa weight[d]	Spleen weight[d]
–	–	10	402 ± 57	353 ± 70[e]	234 ± 44[f]
+	–	10	372 ± 55	52 ± 20	172 ± 58
+	+	12	385 ± 67	284 ± 59[e]	224 ± 63[f]

[a] 1 50 mg/embryo/day on days 15, 16 and 17 of incubation
[b] Measured at the age of six weeks, mean ± SD are given
[c] On day 18 of incubation
[d] Mg/100 g of body weight
[e] $P<0\ 001$ } as compared to CY-treated controls
[f] $P<0\ 05$ }

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Measured as a proportion of standard serum by ELISA

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[b] P<0 01
[c] P<0 05
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[a] 1 50 mg/embryo/day on the days 15 16 and 17 days of incubation
[b] On day 18 of incubation
[c] Mean ± SD are given
[d] P < 0 05 as compared to CY treated controls

TABLE 4 *Chromosome Analyses of Bursa Thymus and Spleen Cells of 6-Week-Old Female Birds[a] Transplanted on Day 18 of Incubation with 5 × 10⁶ Bursa Cells from 18-D Male Embryos For the Bursa, Direct Analysis of Unstimulated Cells Was Applied Thymus and Spleen Cells Were Stimulated with Con A Before the Chromosome Analysis Unstimulated Cultures Represent the Background*

Organ	Mitogen	No of chickens	No of cells analyzed ♂	No of cells analyzed ♀	% donor cells
Bursa	–	7	120	64	65 2
Thymus	Con A	8	0	170	0 0
Spleen	–	7	0	48	0 0
Spleen	Con A	7	8	201	3 2

[a] Treated with CY (1 50 mg/embryo/day) on days 15 16 and 17 incubation

transplantation of 18-d embryonic bursa cells was assessed by determining the weight of the bursa and spleen as well as the production of antibody to HGG and *Brucella* The effects of CY given *in ovo* were similar to those described previously (3–5) Transfer of 18-d embryonic bursa cells induced a significant increase in the gain in the relative weight of bursa and spleen (Table 1) Likewise the specific IgM and IgG class antibody production against HGG and *Brucella* was restored to a normal level (Table 2) These findings indicate that B cell functions were well restored by transplantation of the bursa cells As regards T cell functions, the effect of CY on the PHA and Con A responses of peripheral blood lymphocytes was studied at 5, 7 and 11 weeks after hatching (Table 3) Both responses were significantly ($P < 0\ 05$) decreased in the CY-treated chickens of all age groups Transplantation of 18-day embryonic bursa cells restored these responses to the normal level (Table 3)

Migration and differentiation of bursa cells was assessed by chromosome analysis of bursa, thymus and spleen cells of 6-week-old cell recipients The results given in Table 4 show that the transplanted embryonic bursa cells had homed and proliferated in the bursa The mean percentage of *dividing donor* cells in the bursas of the seven recipients was 65 % thus showing that the majority of the bursa cells were of donor origin though cells of host-derived were also present In contrast, Con A responsive T cells in the thymus remained exclusively of the host origin, no cells of the opposite sex were detected In the spleen, 8 metaphases out of 209 Con A responsive T cells were opposite sex No cells of opposite sex were observed in the unstimulated control cultures

DISCUSSION

According to the chromosome analysis presented, it would seem that 18-d embryonic bursa cells which are capable of a functional reconstitution of the B cell system do not contain T cell precursors No clear evidence was obtained that embryonic bursa cells could differentiate into functional Con A responsive T cells Only a few Con A responsive spleen cells were of opposite sex, but no such cells were observed in the thymus Those few metaphases of opposite sex may represent spontaneous donor cell proliferation in the spleen

The results of this study extend and confirm the previous findings by *Weber & Alexander* (22) They also failed to demonstrate the influx of donor cells from 13–14-d embryonic bursa into the thymus and their differentiation into functional T cells All these data suggest that embryonic bursa cells are already restricted to the B cell development and have lost their capacity to migrate into the thymus and develop into mature T cells It is possible that stem cells are committed for the B cell line already before entering the bursa as suggested by *Weber & Mausner* (24) on the basis of results obtained by transplanting embryonic bone marrow cells into irradiated chicken embryos

On the other hand *Jotereau & Houssaint* (8) and *Le Douarin et al* (12) have observed that 11-d embryonic quail bursa contains cells capable of colonizing 6-d embryonic thymus, which is then attractive for stem cells On this basis it seems probable that in the early stages of bursal differentiation the bursa cells have the ability to differentiate into T cells At that stage, bursa may harbour pluripotential stem cells, which might also explain the induction of

factors on embryonic bursa cells (1) However these findings do not indicate that migration of bursa cells to the thymus is a physiological phenomen since the thymus is attractive for stem cells much earlier than the time at which the bursa contains any stem cells (12)

The present findings reveal that CY affects the T cell dependent immune system in the chicken as shown previously (3–5 19 20) but whether this is due to a reduced T cell level or to a malfunction of individual T cells remains open The mitogenic responses of peripheral blood lymphocytes against PHA and Con A in CY treated chickens are significantly lower as compared to those of untreated chickens Bursa cell transfer induced a clear-cut restoration of the PHA and Con A responses However if bursa cells do not develop into mitogen responsive T cells other cellular mechanisms are needed to explain these observations The lower responses in CY treated chickens may also be due to the effect of CY on monocytes which are shown to be important in the mitogen activation of T cells (16) B-cell deficiency in CY treated birds may also explain the lower responses since the presence of B cells facilitates T cell proliferation after mitogen stimulation (25) Furthermore the recovery of the humoral immune system resulting in a better general condition of the cell recipients might restore the T lymphocyte responses independently by the mechanism by which CY affects T cell functions

Although there is no evidence for bursa-derived T cells on the basis of present results a small T cell subpopulation of bursal origin not detectable with sex chromosome marker used may still exist It must be noted that use of the chromosome marker permits only analysis of a minority of the cells and thus is not the most sensitive technique It can also be argued that bursa-derived T cells do not divide after Con A stimulation and therefore remained undetectable Before the existence of bursa-derived T cells can be ruled out with certainty further studies must be performed using more sensitive techniques for instance bursal and thymic alloantigens within inbred chicken lines (7)

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Migration and differentiation of bursa cells was assessed by chromosome analysis of bursa, thymus and spleen cells of 6-week-old cell recipients The results given in Table 4 show that the transplanted embryonic bursa cells had homed and proliferated in the bursa The mean percentage of dividing donor cells in the bursas of the seven recipients was 65 % thus showing that the majority of the bursa cells were of donor origin though cells of host-derived were also present In contrast Con A responsive T cells in the thymus *remained exclusively* of the host origin no cells of the opposite sex were detected In the spleen 8 metaphases out of 209 Con A responsive T cells were opposite sex No cells of opposite sex were observed in the unstimulated control cultures

DISCUSSION

According to the chromosome analysis presented it would seem that 18-d embryonic bursa cells which are capable of a functional reconstitution of the B cell system do not contain T cell precursors No clear evidence was obtained that embryonic bursa cells could differentiate into functional Con A responsive T cells Only a few Con A responsive spleen cells were of opposite sex, but no such cells were observed in the thymus Those few metaphases of opposite sex may represent spontaneous donor cell proliferation in the spleen

The results of this study extend and confirm the previous findings by *Weber & Alexander* (22) They also failed to demonstrate the influx of donor cells from 13–14-d embryonic bursa into the thymus and their differentiation into functional T cells All these data suggest that embryonic bursa cells are already restricted to the B cell development and have lost their capacity to migrate *into the thymus* and develop into mature T cells It is possible that stem cells are committed for the B cell line already before entering the bursa as suggested by *Weber & Mausner* (24) on the basis of results obtained by transplanting embryonic bone marrow cells into irradiated chicken embryos

On the other hand *Jotereau & Houssaint* (8) and *Le Douarin et al* (12) have observed that 11-d embryonic quail bursa contains cells capable of colonizing 6-d embryonic thymus which is then attractive for stem cells On this basis it seems probable that in the early stages of *bursal differentiation* the bursa cells have the ability to differentiate into T cells At that stage bursa may harbour pluripotential stem cells which might also explain the induction of T cell characteristics by thymic

factors on embryonic bursa cells (1) However these findings do not indicate that migration of bursa cells to the thymus is a physiological phenomen since the thymus is attractive for stem cells much earlier than the time at which the bursa contains any stem cells (12)

The present findings reveal that CY affects the T cell dependent immune system in the chicken as shown previously (3–5 19 20) but whether this is due to a reduced T cell level or to a malfunction of individual T cells remains open The mitogenic responses of peripheral blood lymphocytes against PHA and Con A in CY treated chickens are significantly lower as compared to those of untreated chickens Bursa cell transfer induced a clear-cut restoration of the PHA and Con A responses However if bursa cells do not develop into mitogen responsive T cells other cellular mechanisms are needed to explain these observations The lower responses in CY treated chickens may also be due to the effect of CY on monocytes which are shown to be important in the mitogen activation of T cells (16) B-cell deficiency in CY treated birds may also explain the lower responses since the presence of B cells facilitates T cell proliferation after mitogen stimulation (25) Furthermore the recovery of the humoral immune system resulting in a better general condition of the cell recipients might restore the T lymphocyte responses independently by the mechanism by which CY affects T cell functions

Although there is no evidence for bursa-derived T cells on the basis of present results a small T cell subpopulation of bursal origin not detectable with sex chromosome marker used may still exist It must be noted that use of the chromosome marker permits only analysis of a minority of the cells and thus is not the most sensitive technique It can also be argued that bursa-derived T cells do not divide after Con A stimulation and therefore remained undetectable Before the existence of bursa-derived T cells can be ruled out with certainty further studies must be performed using more sensitive techniques for instance bursal and thymic alloantigens within inbred chicken lines (7)

I wish to thank Professor *Paavo Toivanen* and Dr *Jussi Eskola* for helpful and invaluable criticism Dr *Matti Viljanen* for antibody determinations Mrs *Tuija Kivari* and Mrs *Tuula Eterma* for expert technical assistance and Pharmaceutical Manufacturers *Laake Oy* Turku Finland for gifts of cyclophosphamide

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Acta path microbiol scand Sect C 87 293–300 1979

DIRECT EFFECTS OF *CORYNEBACTERIUM PARVUM* AND BCG ON HUMAN MONOCYTE-MEDIATED TUMOUR CELL CYTOSTASIS *IN VITRO*

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Hammerstrøm J Direct effects of *Corynebacterium parvum* and BCG on human monocyte mediated tumour cell cytostasis *in vitro* Acta path microbiol scand Sect C 87 293–300 1979

Four strains of *Corynebacterium parvum* (Cp) and BCG induced low levels of cytostatic ability to a human tumour cell line in human monocytes when added directly to conventional monocyte cultures The cytostatic ability induced by mediators from autologous lymphocytes stimulated with the same agents was greater than that produced by direct addition to monocytes BCG was more efficient in stimulating lymphocyte DNA-synthesis and lymphokine release than any of the Cp strains tested In order to test the influence of contaminating adherent lymphocytes on the direct induction of cytostasis monocyte cultures of > 99 9% purity were prepared by adherence purification Cp induced low levels of cytostatic ability in such highly purified monocytes when added directly to the monocytes Addition of BCG and *Candida albicans* had an adverse effect on the cytostatic ability of purified monocytes A morphological study of Cp interaction with purified monocytes was performed Cp but not BCG would seem to be able to induce low levels of cytostatic ability in human monocytes without lymphocyte cooperation Human monocyte activation by the more effective lymphokine pathway is more efficiently triggered in vitro by BCG than by Cp

Key words Monocytes human *Corynebacterium parvum* BCG lymphokines cytostasis

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Accepted as submitted 8 iii 79

Attempts to recruit the mononuclear phagocyte in the eradication of human cancer by means of nonspecific active immunotherapy have met with

...uch less is known about the effect of these agents on human monocytes and macrophages We have recen...

... rendered cytotoxic to tumour cells by Cp and possibly also by BCG without lymphocyte cooperation Tumour regression can be induced in T-cell-depleted or congenitally athymic nude mice by Cp (12 18 2) and tumouricidal macrophages can be recovered from the peritoneum of such mice (2). BCG can also induce tumour regression in nude mice (17) *In vitro* Cp and ...

... walls added to guinea pig macrophage cultures increase membrane incorporation of ^{14}C-glucosamine in the cells (13) Several problems complicate the interpretation of such data Both Cp and BCG can activate B-cells (23 19) which in turn are capable of lymphokine synthesis (22) and this pathway may be responsible for the macrophage activation and tumour regression observed in T-cell deficient mice In *in vitro* experiments the presence of a small percentage of adherent lymphocytes in

migration in developing myeloid and lymphoid systems Lancet *2* 658–659, 1967
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TABLE I *Effect of D rect Add tion of Cp (CN 6966) to Monocytes on Monocyte mediated Cytostasis*

Monocyte culture time before Cp addition	Monocyte treatment	Cpm in target cells as % of target cell control	CI	Lymphocyte[a] contamination	p
3 days	HS M	104 4 ± 4 1	0	4 3%	
	CN 6966	85 8 ± 5 7	18 9 ± 3 0		<0 004
7 days	HS M	55 6 ± 10 5	0	0 4%	
	CN 6966	42 9 ± 9 0	20 4 ± 8 9		<0 004

Mean ± S E M
n = 20 at 3 days n = 8 at 7 days
[a] ANAE – negative cells

dose response curves shown in Fig 1 obtained in parallel experiments with monocytes from two donors At 50 µg/ml and higher doses considerable cell clumping and some detachment were observed All further experiments were performed with 5 µg/ml CN 6966 did not induce significant DNA synthesis in the monocyte monolayers either 24 or 72 hours after addition of Cp (data not shown)

Comparison of Four Cp Strains and BCG in Induct on of Mon

than that induced by lymphokines

activities of Cp have been described in animal experiments (14) a protocol was designed in which the effects of four Cp strains and BCG could be compared

The

onocyte mediated cytostatic activity induced by

All Cp strains induced enhanced cytostasis by direct addition to monocytes (Fig 2 grey columns) CN 5888 which is largely inactive in animal *in vivo* systems induced a slightly lower response than CN 6966 and CN 5936 (p < 0 063) as did also BCG It should be noted that CN 6134 is not directly comparable to

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Lymphokine supernatants of autologous lymphocytes stimulated by the same agents (Fig 2 open columns) were significantly (p < 0 031) more effective in promoting monocyte cytostatic activity

The difference between the CI produced by direct addition to monocytes and that produced by lymphokines was greatest for BCG the poorest direct stimulant and least for CN 5936 the best direct stimulant BCG induction of lymphokine release from human lymphocytes was clearly superior to all Cp strains (p = 0 031)

DNA synthesis in Lymphocytes Stimulated with Four Cp Strains and BCG

Lymphocyte ³H methyl TdR incorporation in response to the four Cp strains and BCG was tested in

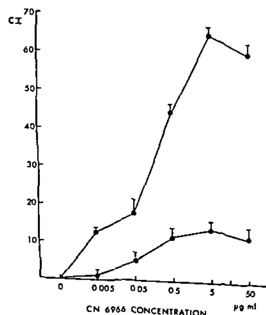


Fig 1 Dose response relationship of the cytosta ndex CI

were added on day 4 Mean ± SD of triplicate cultures

conventional mononuclear phagocyte cultures makes it difficult to ascribe effects produced by direct addition of agents to such cultures to a direct action on mononuclear phagocytes, unless lymphocyte contribution can be ruled out by appropriate controls

This study reports the results of experiments designed to investigate whether Cp and BCG can enhance the cytostatic ability of human monocytes *in vitro* without lymphocyte cooperation and to compare the efficiency of four Cp strains and BCG in human monocyte activation

MATERIALS AND METHODS

Human monocytes were separated from defibrinated venous blood of healthy Pirquet positive volunteers by Ficoll/Isopaque centrifugation and plastic adherence as described in (6) and (7) Monocyte monolayers consisted of > 90% monocytes at the start of culture as determined by phagocytosis of *Candida albicans* (6) α naphtyl esterase staining (ANAE) (8) and phase contrast microscopy All cells were cultured in RPMI 1640 (Gibco Bio Cult Glasgow Scotland) supplemented with 25% pooled human AB Rh + serum 0 1 mM l glutamine and 40 μg gentamycin per ml (HS M) Monocytes were cultured in 24 well tissue culture plates (Costar 3524 Costar Cambridge Mass USA well size 17 8 × 16 mm) in 0 5 ml HS M

Human lymphocytes The non adherent cells aspirated after 90 min incubation of mononuclear blood cells on plastic were adjusted to 10^6 cells/ml and cultured as described in (7) Supernatants for lymphokine experiments were harvested after 3 days of lymphocyte culture as described in (7) DNA synthesis in lymphocyte cultures was assayed after 5 days of lymphocyte culture as described in (7)

Corynebacterium parvum Strains CN 5888 CN 6966 and CN 5936 were supplied by Dr *C Adlam* Wellcome Research Laboratories Kent England and cultured and supplied as a freeze dried preparation by Dr *A Dalen* Dept of Microbiology University of Trondheim Freeze dried Cp was killed by UV irradiation suspended in RPMI 1640 at 1 mg/ml dry weight and stored at −20° C Strain CN 6134 (Coparvax Wellcome lot BA 4049) was obtained in formalin killed form washed 3 × in 0 9% NaCl to remove the thiomersal preservative freeze dried and suspended and stored in the same way as the other strains Lymphocyte stimulation was performed with 2 5 μg Cp/ml which is the optimal dose (7) *BCG* was obtained as a freeze dried live vaccine from Statens Seruminstitut Copenhagen Denmark One ampoule was suspended in 2 5 ml Sautons medium and 40 μl of this suspension which contains about 10^6 bacilli was chosen as 1 standard unit (1 std) Lymphocyte stimulation was performed throughout with 1 std /ml which is the optimal dose (21)

Candida albicans was supplied as a heat killed preparation from Dept of Microbiology University of Trondheim washed 6 × in isotonic saline and stored at −20° C

Addition of Cp BCG Candida albicans and lymphocyte supernatants to monocyte cultures Monocyte cultures were shaken vigorously the medium with nonadherent cells was aspirated and 0 25 ml fresh HS M was added Cp BCG or Candida diluted to twice the desired concentration in 0 25 ml HS M or 0 25 ml filtered lymphocyte supernatants was then added and the cultures were incubated for 24 hours before assay

Assay for monocyte mediated cytostasis The human cell line NHIK 3025 originating from a carcinoma in situ of the cervix (16) was used as target cells as described in (7) The medium was aspirated from monocytes after the plate was shaken and 10^4 target cells were added in 0 5 ml fresh HS M resulting in effector/target ratios of approximately 10 1 Target cell DNA synthesis was determined by adding 1 μCi methyl ^3H TdR (Sp act 5 μCi/mM Radiochemical Centre Amersham England) for the last 5 hours of a 24 hour coculture period and harvesting the cultures as described in (6) The results are expressed as cytostatic index (CI) which describes the enhancement in monocyte mediated cytostasis produced by the treatment relative to the cytostatic effect of monocytes cultured in HS M

$$CI = 100 - \frac{\text{cpm (treated monocytes + NHIK 3025)}}{\text{cpm (untreated monocytes + NHIK 3025)}} \times 100$$

Cpm in monocytes alone was not subtracted as this did not exceed 5% of cpm in target cells cultured alone Target cell cpm in controls without monocytes was 57731 ± 2660 (mean ± SEM of all experiments included)

In some experiments the results are also given as cpm in target cells cultured with monocytes as % of cpm in target cells cultured in medium alone

$$\frac{\text{cpm (monocytes + NHIK 3025)}}{\text{cpm (NHIK 3025)}} \times 100$$

Statistics The figures given are mean ± SEM of n experiments performed in triplicate P values were obtained by Wilcoxon s signed rank test

RESULTS

Effects of Direct Addition of CN 6966 on Monocyte mediated Cytostasis

Direct addition of CN 6966 to monocytes 24 hours before target cell addition induced cytostatic ability in monocytes cultured for 3 or 7 days *in vitro* before treatment (Table I) Allowing a 72 hour induction period by adding CN 6966 at day 1 of monocyte culture resulted in variable and generally low levels of cytostatic enhancement (data not shown)

The cytostatic activity induced varied considerably between the cell donors as exemplified by the

TABLE 4 *Purification Procedure to Obtain Highly Purified (>99 9%) Monocytes*

Time of Monocyte Culture	Shaking + aspiration of non adherent cells	Washing with RPMI 1640	Medium change	Trypsin exposure (0 25%, 5 min)
90 min	4 ×	3 ×	1 ×	–
Day 1	1 ×	–	1 ×	–
Day 3	1 ×	–	1 ×	–
Day 4	4 ×	2 ×	1 ×	1 ×
Day 7	1 ×	–	1 ×	–

produced as estimated by direct microscopy, phagocytosis of *Candida albicans* and ANAE-staining The cells were 99% viable as determined by trypan blue exclusion but the number of adherent cells at the time of activation was only about 20% of the value at start of culture, thus indicating that a rather large number of monocytes is also removed by this protocol Direct Cp addition to these highly purified monocytes produced some enchancement of cytostasis while BCG reduced it in all experiments (Fig 3) *Candida* phagocytosis also produced an adverse effect Dose response experiments with these highly purified monocyte cultures indicated an optimal Cp dose of 5–25 µg/ml lower doses gave inconsistent results and higher doses cell detachment and death BCG in doses from 1/100 std to 10 std always produced impairment of monocyte mediated cytostasis which increased with dosage and was accompanied by some cell detachment at the highest dose level Supernatants of treated or untreated monocytes enhanced target cell proliferation in all experiments (Table 5)

TABLE 5 *Lack of Cytostatic Activity of Supernatants of Monocyte Cultures Treated with Cp BCG or* Candida

Monocyte treatment	Cpm in target cells as % of target cell control
HS M	113 9 ± 9 0
CN 5888	115 4 ± 8 0
CN 5936	118 6 ± 7 6
BCG	111 3 ± 3 4
Candida	121 7 ± 3 7

Highly purified monocytes Cp BCG or Candida added on day 7 Fresh HS M added on day 8 Monocyte supernatants collected on day 9 and 0 5 ml of cell free supernatant added to target cells freshly plated in 0 1 ml HS-M Target cell DNA synthesis assayed after 24 hour exposure to monocyte supernatants
Mean ± S E M n = 3

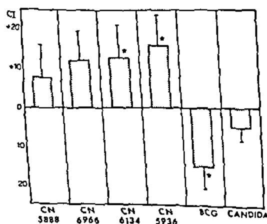


Fig 3 Cytostatic index (CI) induced in highly purified (> 99 9%) monocyte cultures by direct addition of four Cp strains (5 µg/ml) BCG (2 std /ml) and *Candida albicans* (2×10^6/ml) on day 7 of monocyte culture Target cells were added on day 8 Mean ± S E M n = 8 *p < 0 031 as compared to untreated monocytes

Morphological Studies

Highly purified monocytes (Fig 4a) ingested *Candida albicans* quickly and avidly (Fig 4b) while Cp was bound more slowly to the cells Many unbound organisms could be seen floating freely between the cells 15 minutes (Fig 4c) after addition of Cp In live preparations organisms were observed to touch the cell membrane without attaching However the number of unbound Cp diminished greatly during 24 hours Cp attachment occurred initially mainly in the periphery of the cell (Fig 4d) After 24 hours organisms could no longer be seen in the cells and there was some vacuolisation in the perinuclear area indicating that the attached

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c

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TABLE 2 *Classification and Activity in Animal Experiments (3 4 14) of the Cp strains Employed*

Wellcome classification	CN 6134	CN 5936	CN 5888	CN 6966
Johnson and Cummins classification (10)	*P* [a] *acnes I*	*P acnes II*	*P granulosum*	*P avidum I*
National collection of type cultures NCTC		10390	10387	
Antitumour activity *in vivo* (mouse)	+ (14)	+ (14)	– (14)	
Increase in spleen weight (mouse)	+ (14)	+ (14)	– (14)	+ (4)
Macrophage activation *in vivo* (mouse PEC)	+ (14)	+ (14)	± (14)	
Direct macrophage activation *in vitro* (mouse PEC)	+ (3)		+ (3)	

[a] *Propionobacterium*

lymphocytes from the experiments shown in Fig 2 All Cp strains induced lymphocyte DNA synthesis (Table 3) CN 5936 induced a significantly greater response than the other Cp strains and BCG was superior to all Cp strains CN 5888 was almost as effective as the other »active« Cp strains

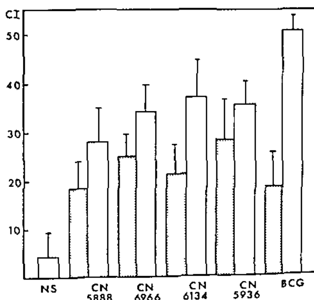


Fig 2 Cytostatic index (CI) induced in monocyte cultures by direct addition of Cp 5 µg/ml or BCG 1 std / ml (■) or by supernatants of autologous lymphocytes stimulated with Cp 2 5 µg/ml or BCG 1 std /ml (□) NS indicates supernatants of non stimulated lymphocytes All monocyte treatments were tested in parallel experiments on day 3 of monocyte culture in six separate experiments in triplicate Mean ± S E M

Influence of Lymphocyte Depletion on Direct Activation of Monocytes

The monocyte cultures used in the experiments described above contained 2–6 % adherent lymphocytes at the time of Cp or BCG addition at day 3 Lymphocyte adherence to plastic which is trypsin sensitive in contrast to monocyte adherence decreases after a few days in culture and almost all lymphocytes become detached after one week in unstimulated cultures These characteristics were used for further purification of monocytes By utilizing the combination of prolonged culture washing aspiration of non adherent cells and trypsin treatment given in Table 4 monocyte cultures with less than 0 1 % lymphocytes were

TABLE 3 *Methyl ³H TdR Incorporation in Lymphocytes Stimulated with Four Cp Strains and BCG*

Lymphocyte stimulation	Cpm
None	1 973 ± 713
CN 5888	48 303 ± ? 448
CN 6966	53 279 ± 6 138
CN 6134	51 257 ± 8 072
CN 5936	68 761 ± 8 876[a]
BCG	85 999 ± 10 011[b]

2 5 µg/ml Cp or 1 std /ml BCG was added at start of culture and methyl ³H TdR incorporation was assayed on day 5 of lymphocyte culture Mean ± S E M n 6

[a] $p = 0.016$ as compared to other Cp strains

[b] $p < 0.05$ as compared to CN 5888 and CN 6966

membrane ruffling and a tendency to cluster formation (Fig 4e)

DISCUSSION

Exposure of conventional human monocyte cultures to Cp or BCG induced a modest enhancement of cytostatic activity This occurred both with relatively undifferentiated 3-day old monocytes which without treatment are not cytostatic in this protocol and with differentiated 7-day old cells which by themselves are cytostatic This is in accordance with results obtained with oil induced mouse peritoneal macrophages (3) These like human monocytes could also be activated »directly« by CN 5888 which is inactive *in vivo* in the mouse (14)

The problem of residual adherent lymphocytes mediating such effects has not received sufficient attention in many reports concerning »direct« *in vitro* activation of macrophages Since in every instance lymphokine rich supernatants of stimulated autologous lymphocytes induced higher levels of cytostatic ability in the monocytes than the direct addition of the same agent to the monocytes lymphokine release from adherent lymphocytes might be responsible for the activation induced by the latter procedure

Viable monocyte monolayers of high purity were produced by a careful timing of washing aspiration and trypsin treatment procedures during the first week of monocyte culture In these cultures CN 6966 CN 6134 and CN 5936 still induced some enhancement of cytostatic activity thus indicating that »active« Cp strains may promote monocyte mediated tumour cell inhibition directly without lymphocyte cooperation The cytostatic activity was not mediated by stable supernatant factors released from monocytes The activation produced by BCG addition to conventional monocyte cultures is probably mediated by contaminating lymphocytes since BCG consistently reduced the cytostatic activity of highly purified monocytes

Phagocytosis has been reported to induce release of unstable or serum inhibited mediators from mononuclear phagocytes with possible potential for inhibitory action on tumour cells such as superoxide anion (11) prostaglandins (9) and lysosomal enzymes (5) The observation that BCG or Candida phagocytosis had an adverse effect on monocyte mediated cytostasis indicates that metabolic or secretory events related to phagocytosis and break down of ingested material are not important *per se*

The purification procedures employed in this study are deliberately simple in order to ensure minimal interference with monocyte function However they do remove a relatively large fraction of ANAE + phagocytic cells together with the lymphocytes The diminished response to Cp obtained in the highly purified populations may thus be due partly to lower effector cell density in the purified cultures or to the removal of a less adherent monocyte subpopulation capable of responding to Cp with higher cytostatic ability than the remaining cells The latter hypothesis was not supported by the control experiments performed The cells removed by the trypsin step at day 4 were collected plated in new wells and purified (95 7% ANAE + cells) by washing Cp induced about the same levels of cytostatic enhancement in these cultures as in parallel highly purified cultures Earlier experiments with varying effector/target cell ratios (20) indicate that the reduced response of purified monocytes to Cp is not explained by the lower effector/target cell ratios in the purified cultures

It would thus seem that activation of cytostatic ability in human monocytes by Cp is effected mainly by monocyte activating lymphokines released from lymphocytes stimulated with Cp although in addition Cp is able to induce low level cytostatic ability in monocytes without lymphocyte cooperation BCG in optimal doses is superior to Cp as an inducer of lymphocyte proliferation and lymphokine release This may of course be related to the immunization status of the donor population BCG does not seem to be able to activate human monocytes to cytostasis without lymphocyte cooperation

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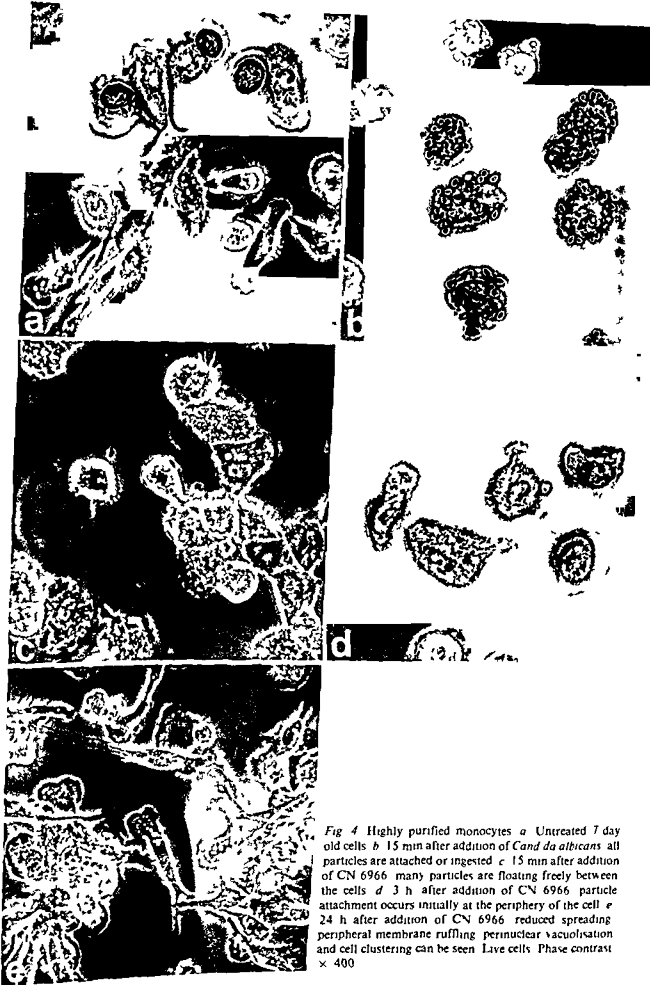


Fig 4 Highly purified monocytes *a* Untreated 7 day old cells *b* 15 min after addition of *Candida albicans* all particles are attached or ingested *c* 15 min after addition of CN 6966 many particles are floating freely between the cells *d* 3 h after addition of CN 6966 particle attachment occurs initially at the periphery of the cell *e* 24 h after addition of CN 6966 reduced spreading peripheral membrane ruffling perinuclear vacuolisation and cell clustering can be seen Live cells Phase contrast × 400

a path microbiol scand Sect C 87 301–306 1979

CHARACTERIZATION OF HEAT ELUATES OF HUMAN MALIGNANT TISSUES

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Wesenberg F Characterization of heat eluates of human malignant tissues Acta path microbiol scand Sect. C 87 301–306 1979

Eluates of 13 malignant and 17 normal tissues were prepared at 56° C using the continuous flow technique *Albumin* was detected in all the eluates IgG IgA C3 or haptoglobin were detected in most of the malignant and some of the normal tissues Carcinoembryonic antigen β_2-microglobulin α_1 antitrypsin or α_1 antichymotrypsin were detected in some of the eluates of the malignant tissues only IgM IgD C1q C4 C1 INH α macroglobul n β l [illegible]
not detected in an [illegible]
was similar in ext [illegible]
non specific bindi[illegible]

Key words Human cancer eluates immunoglobulins complements proteins

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Eluates of human malignant tissue obtained using differing techn[illegible]
lin[illegible]
Or[illegible]
he [illegible] have also been detected We have previously shown (14) that eluates made at 37 45 and 56° C of 13 homogenized human malignant tissue samples contain larger amount of IgG than eluates of normal tissue and apparently 2 of the carcinomas contained predominantly non specifically bound IgG whereas 2 other carcinomas contained both specifically and non specifically bound IgG In the experiments presented here the same eluates were investigated for the presence of other serum proteins and by comparing the ratio of the concentration of albumin to the concentration of IgG in extracts and eluates evidence was obtained that non specifically bound IgG was present in all normal tissues and also in a fifth carcinoma

MATERIALS AND METHODS

Extracts and Eluates

Extracts and heat eluates of 2 fibrosarcomas 11 carcinomas from the colon breast bronchus ovary endometrium cervix and bladder of which 3 included both primary and secondary deposits and a total of 17 various normal tissues were the same as used previously having been stored at −25° C (12 14) The tissue had been obtained at autopsies and had been gently minced with scissors homogenized for 1 min in a Servall Omni Mixer and centrifuged at 20 000 × g for 20 min The supernatant beneath the lipid layer was called the extract Three grams of wet tissue sediment was resuspended in PBS and placed between glass fibre filters and glass wool in a short glass column The tissue sediment was washed at +4° C and eluted at 37 45 and 56° C successively in a continuous upward flow of PBS by submers[illegible]

[illegible] there was no loss of proteins during concentration procedure

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TABLE 1 *Some of the Proteins Identified in the 56° C Eluate of Human Malignant and Normal Tissues*

Eluates of tissue	Antiserum to					
	IgA	C3	Haptoglobin	Antichymotrypsin	Antitrypsin	β_2-microglobulin
Carcinoma of						
A uncertain origin	+	+	−	−	−	+
B the colon	+	+	+	+	+	+
C the bronchus	+	+	+	+	+	−
D »	+	−	−	+	+	+
E »	+	−	−	−	−	+
F the breast	−	−	+	+	+	−
G »	+	−	−	−	+	+
H the ovary	−	−	+	−	−	−
I the cervix	+	−	+	−	−	+
J the endometrium	−	−	−	−	−	+
K the bladder	+	+	+	+	+	+
Fibrosarcoma						
L	+	+	+	+	+	−
M	−	−	−	−	−	−
Normal organs						
Liver	−	−	−	−	−	−
Spleen	−	−	−	−	−	−
Muscle	−	−	−	−	−	−
Lung	+	−	−	−	−	−
Stomach	+	−	−	−	−	−
Kidney[a]	+	+	+	−	−	−

[a] Kidney from individuals aged 50–60 years
\+ present
− – not present

precipitation lines appeared mostly when the 56° C eluate was used and none of the lines found in the 37° C and the 45° C eluates were absent *in the 56°* C eluates One of the lines corresponded *to IgG and* one to albumin both identified using *specific* antiserum in an intermediate gel Eluates of normal kidney from young individuals (aged 20 to 30 years) and eluates of normal spleen and liver from individuals of various ages (aged 20 to 60 *years*) displayed only one line corresponding to *albumin* Eluates of normal kidney from older individuals (aged 40 to 60 years) and eluates of normal stomach lung and muscle from individuals of various ages displayed 3 to 8 lines *corresponding to* those obtained using eluates of malignant tissue

CIE of extracts or eluates of malignant *tissue* against antiserum to tumour haemagglutinins showed 1 or 2 precipitation lines corresponding to the lines formed by IgG *and* IgA Similar results were obtained using extracts or eluates of normal tissue

Precipitation in Agar

The following results were obtained using the *double* diffusion test in agar of eluates against *differing* antisera to human proteins Albumin was *found in all* eluates None of the eluates of normal kidney from younger individuals nor of *normal* spleen or liver contained IgG IgG was detected in most of the eluates prepared at 37 and 45° C and in *all* the eluates prepared at 56° C from the other tissues Further as shown in Table 1 IgA C3 and *haptoglobin* were detected in some of the 56° C eluates β_2 microglobulin α_1 antitrypsin *and* α_1 antichymotrypsin were present in some of the eluates of malignant tissue and were not detected in any of the eluates of normal tissue The same results were obtained both when using primary tumour *tissue and* when using their secondary deposits

CEA was only detected *in the extract* and in the 56° C eluate of the carcinoma of the colon and the *carcinoma* of uncertain origin

Antisera to the following proteins did not

Sera

Sera were obtained from 2 of the patients suffering from malignant disease and from 2 of the patients from whom a total of 5 normal organs were obtained

Antisera to a pool of human serum (anti PHS) were the same as used previously (13) They had been raised in rabbits by subcutaneous immunization with a mixture of PHS and Freund s complete adjuvant

Antisera to rabbit erythrocytes agglutinated by a pool of tumour extracts (abbr antiserum to tumour haemag glutinins) were produced by the immunization of 2 rab bits with their own erythrocytes agglutinated by the ex tracts as described previously (13)

Antisera to human α_1 foetoprotein haptoglobin α_2 macroglobulin α_1 antichymotrypsin α_1 antitrypsin fi brinogen β_2 lipoprotein C3/C3c (β_1C β_1A globulin) C4 (β_1E globulin) C1 inactivator (C1INH) C1q compo nent IgG IgM and IgD were purchased from Behring werke AG Marburg Lahn West Germany Antisera to human IgA carcinoembryonic antigen (CEA) albumin and β_2 microglobulin were purchased from Dako immunoglobulins Copenhagen The specificity of the antisera was confirmed by double diffusion test in agar using mixtures of and isolated antigen preparations

Precipitation

The double diffusion test was performed in 1 per cent agar in PBS as previously described (13) When using antiserum to C1q component 0 5 per cent agarose in PBS was used and sera extracts or eluates were first applied and the antiserum was applied 2 h later

Crossed Immunoelectrophoresis (CIE)

CIE was performed using the micro technique descri bed by *Weeke* (11) The first dimension was carried out with 10 V/cm for 60 min at + 10° C The slab was then transferred to a 5 × 5 diapositive photoglass placed along the centre line and the antibody containing gel was poured onto the remaining parts of the plate Electropho resis in second dimension with 2 V/cm at + 10° C was carried out overnight (16–22 h) Varying amounts of antisera extracts or eluates were used to obtain optimal precipitation conditions In some experiments an inter mediate gel containing specific antisera to different proteins was used

Quantification of IgG and Albumin

IgG and albumin in sera extracts and eluates were quantified using single immunodiffusion technique The immunodiffusion plates were purchased from Behring werke AG

RESULTS

CIE

The patterns using sera and extracts tested by CIE against anti PHS were similar (Fig 1) Similar results were obtained using extracts from normal and malignant tissue

Characteristic results obtained using eluates of malignant tissue are shown in Fig 2 Three to 8

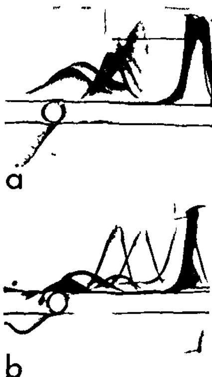


Fig 1 Crossed immunoelectrophoresis of a) pooled human serum and b) extract of a carcinoma of the bronchus Antiserum to pooled human serum in the gel

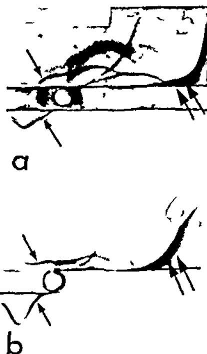


Fig 2 Crossed immunoelectrophoresis of a) 56° C eluate of a carcinoma of the colon and b) 56° C eluate of a carcinoma of the bronchus Antiserum to pooled human serum in the gel

→ IgG

⇉ albumin

Gupta & Morton (3) using 15% NaCl at 37° C as the elution procedure of 1 sarcoma and 3 carcinomas However *Gupta & Morton* (3) in addition detected trace amounts of IgM in 2 out of 5 eluates of malignant melanoma *Vanky et al* (10) found binding of radionated anti Ig reagent assayed by acid elutable radioactivity in 18 out of 44 tumour cell suspensions and found the same amount of IgG and IgM in the 16 tumours tested for these proteins Using immunofluorescence techniques IgM has been detected in sections of human tumours but it has been difficult to determine to what extent this IgM is associated with the malignant cell (reviewed in 15)

In addition to immunoglobulins albumin C3 haptoglobin CEA β-microglobulin and the 2 antiproteases α_1 antitrypsin and α_1 antichymotrypsin were detected in the eluates This is in line with the results obtained by *Thunold et al* (9) who found albumin, and *Romsdahl & Cox* (7) who found albumin transferrin hemopexin and the same 2 antiproteases *Cotropia* (1) using low pH eluates of human acute myelogenous leukemia blasts found IgG and the 2 antiproteases In contrast to the results presented here *Thunold et al* (9) could not detect C3 and *Romsdahl & Cox* (7) could not detect C3 microglobulin and haptoglobin in the eluates *Cotropia* (1) on the other hand detected α macroglobulin and by using the direct immunofluorescent test, IgM was detected on the blasts

Of special interest is the data reviewed by and the finding of *Israël & Edelstein* (6) that the concentration of antiprotease and haptoglobin increased in sera from cancer patients b t the - -

α

d

specifically bound or adsorbed to the malignant cells since the proteins of higher concentration in the extra cellular fluid will be more easily detected in the eluates than other proteins of lower concentration When adsorbed to the tissue these proteins could influence the immune response *in vivo* as shown *in vitro* (6) Some of the proteins could also be synthesized in the tissue as shown by *Hurliman et al* (5) who found synthesis of IgG C3 lactoferrin α and β-globulins *in vitro* from dysplastic and neoplastic human mam- -

al

w

u t a et al (9) *Gupta & Morton* (3) did not detect any immunoglobulins but did not look for other proteins in eluates of kidney lung and muscle However the results presented here show that eluates of normal lung muscle and stomach in addition to albumin contain IgG and for some cases also IgA Further eluates of normal kidney from older individuals in addition contain C3 and haptoglobin This indicates that normal cells may adsorb proteins to their surface rather strongly, since most of them are not eluted at lower temperature than 56° C *Romsdahl & Cox* (7) reported that they isolated all the proteins found in sarcoma eluates with the exception of IgA from the liver from the same patient The question therefore arose whether there is only a quantitative difference or also a qualitative difference between eluates of malignant tissue and that of normal tissue However the present data cannot answer this question Further experiments are therefore necessary to clarify this point

The results presented here are in overall agreement with the results presented by the other authors mentioned The small differences discussed could partly be due to the different elution techniques used and also that some of the proteins are not associated with all the tumours

CEA has been of some value in the diagnosis and the monitoring of several neoplastic disorders (for review see refs 4 and 8) It was first detected in extracts of malignant tissues (2) and it is therefore of interest that also eluates of malignant tissues contain CEA as shown here This implies that other tumour associated antigens may also be eluted from malignant tissues and may recombine with the eluted antibodies

We have previously shown (13) that antiserum to tumour haemagglutinins from extracts of one tumour contained detectable antibodies to IgG and IgA Similar results were obtained in this study using a pool of tumour extracts and no tumour specific proteins were found

The results obtained using CIE and the ratio of albumin to IgG in serum and extracts support our previous interpretation that the extract is representative for the fluid of the extracellular compartment of the tissue (14)

Using the extracts and the corresponding eluates of all the normal tissue and of 3 of the malignant tissues the ratio of albumin to IgG was similar This is in accordance with data reported previously (14) The IgG associated with these normal and malignant tissues are most likely non specifically adsorbed However the demonstration of reduced ratio of albumin to IgG indicates that the IgG are bound to tumour associated antigens or to Fcγ receptors associated with the tissues as discussed previously (12)

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He thanks Miss *Wibecke Aasnæs* and Mrs *Turid Tynning* for their skilful technical assistance

TABLE 2 *Ratios of Concentrations of Albumin to Concentrations of IgG in Extracts and Eluates of Human Malignant and Normal Tissue*

Tissues	Ratio		Reduction of ratio[a]
	Extract	Eluate	
Carcinoma			
A uncertain origin	2 7	1 1	2 45
B the colon	1 2	0 7	1 71
C the bronchus	1 4	1 2	1 17
D »	1 7	0 4	4 25
E »	1 3	1 1	1 18
F the breast	4 2	3 6	1 17
G »	5 5	2 3	2 39
H the ovary	2 0	0 6	3 33
I the cervix	0 9	0 6	1 50
J the endometrium	2 1	0 8	2 63
K the bladder	1 1	0 3	3 70
Fibrosarcoma			
L	3 0	1 3	2 30
M	1 7	1 1	1 55
Normal tissue			
Muscle	2 6	2 9	0 89
Lung	2 4	2 4	1 0
Kidney	2 2	2 7	0 82

[a] Calculated by $\frac{\text{Ratio in extract}}{\text{Ratio in eluate}}$

precipitate any of the eluates α_1 foetoprotein IgM IgD C1q C1 INH C4 α_2 macroglobulin fibrinogen and β lipoprotein

Ratio of Albumin to IgG

Since albumin was detected in all the eluates the concentration of this protein was determined in sera extracts and the 56° C eluates By using the results obtained previously (14) the ratio of the concentration of albumin to the concentration of IgG was calculated The ratio was similar in the serum and corresponding extract both of normal and malignant tissue Using the eluates of normal organs the ratio was similar or slightly increased when compared to the extract (Table 2) Ten of the tumours had a reduced ratio of albumin to IgG ratio in the eluates compared to that in the extracts By calculation (see footnote Table 2) the reduction of the ratio varied from 1 50 to 4 25 Of particular interest is the results obtained using the 2 tumours B and I in that these tumours also showed a reduced ratio of the titre of agglutinins to the concentration of IgG (14) In 3 of the eluates (C E and F) the ratio of albumin to IgG was similar to that in the extracts Two of these eluates (C and F) also had a constant ratio of agglutinins to IgG (14) in the extracts and eluates No difference was found either when using primary tumour or their secondary deposits

DISCUSSION

Eluates of homogenated washed human malignant and normal tissues contain differing serum proteins but more proteins are eluted from the malignant tissue than from the normal tissue This conclusion is based on the results obtained previously (14) which show that more IgG was eluted from malignant than from normal tissue and the data presented here together with those reported by others (1 3 7 9 10) The results obtained here show that most of the eluates of malignant tissue contain IgA in addition to IgG IgM and IgD were not detected This is in line with the results obtained by *Thunold et al* (9) who also used heat eluates of human carcinomas by *Romsdahl & Cox* (7) who used low pH eluates of human sarcomas and by

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IMMUNOHISTOCHEMICAL DEMONSTRATION OF INTRACELLULAR IMMUNOGLOBULIN IN FORMALIN FIXED, PARAFFIN EMBEDDED SECTIONS, AS STAINING METHOD IN DIAGNOSTIC WORK

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Clausen P P Jacobsen M Johansen P & Thommesen N Immunohistochemical demonstration of intracellular immunoglobulin in formalin fixed paraffin embedded sections as staining method in diagnostic work Acta path microbiol scand Sect C 87 307–312 1979

52 specimens from 34 patients with multiple myeloma and 3 cases with reactive plasmacytosis were stained by the 2 step labelled or 3 step unlabelled immunoperoxidase (IP) technique for demonstration of intracellular immunoglobulin in order to test the utility of these methods as staining methods in routine diagnostic work The specificity of the methods was high as agreement between IP staining and serumanalysis for M-component was achieved in 27 out of 28 cases where complete serumanalysis were available Interpersonal variation in interpretation of the staining results was low With optimal dilutions of antisera the evaluations of the examiners were concordant in 51 out of 52 specimens By comparing the different tissue specimens the best result was obtained with iliac bone biopsies both concerning IP staining and morphological preservation No difference was observed between results obtained by the 2 step labelled and 3 step unlabelled IP technique

Key words Immunoperoxidase intracellular immunoglobulin multiple myeloma

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In 1974 *Ta lor & R* (6) a labelled monstra paraffin embedded material

The specificity of the method has later been confirmed by *Taylor & Mason* (7) using the technique on paraffin sections from multiple myeloma and related conditions and by *Pinkus & Said* (4) using an unlabelled 3-step IP technique in several cases of multiple myeloma and macroglobulinaemia

The objectives of this report have been to investigate the utility of the 2-step and 3-step techniques as staining methods in routine diagnostic work by evaluating specificity of the methods interpersonal variation in the interpretation of the staining results and the significance of the character of the tissue specimens

MATERIAL AND METHODS

The material consists of 41 surgical biopsy specimens (36 bone marrow clots and 5 iliac crest needle bone marrow biopsies (Radner)) from 34 cases with either verified or suspected multiple myeloma and from 3 cases with increased number of plasma cells in the bone marrow without any serological or clinical signs of multiple myeloma From 11 of the 34 cases with multiple myeloma further autopsy material has been

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TABLE 1 *Results of Immunohistochemical Demonstration of Intracellular Immunoglobulin Compared with Immunochemical Serum Analysis*

Case no	Histology no		Tissue	IP	Serum or urinary analysis
IgG					
1	Hv	3510/76	MC	IgG L	IgG L
2	–	3108/77	MC	IgG K	IgG
3	–	2446/76	MC	IgG K	IgG K
4	–	6031/76	MC	IgG K	K
5	–	3261/77	MC	IgG K	IgG K
	–	275/77	Autopsy	IgG K	IgG k
6	–	2291/76	MC	IgG k	IgG K
	–	3470/76	MC	IgG K	IgG K
7	–	6614/76	MC	IgG k	IgG K
8	–	6737/76	MC	IgG K	IgG
	–	553/76	Autopsy	IgG K	IgG
9	–	3226/77	MC	IgG L	IgG L
	–	473/77	Autopsy	IgG L	IgG L
10	–	3924/77	MC	IgG K	IgG k
11	–	5579/77	MC	IgG K	IgG
	–	503/77	Autopsy	IgG K	IgG
12	Ålb	C 12377/77	MC	IgG K	IgG K
13	–	C 9728/77	MC	IgG k	IgG k
	–	236/77	Autopsy	IgG K	IgG k
14	–	C 14559/77	MC	IgG K	IgG K
15	–	C 10940/77	MC	IgG L	IgG L
16	–	C 884/77	ICB	IgG K	IgG K
17	–	C 7457/77	ICB	IgG K	IgG k
18	–	C 18996/75	MC	IgG L	IgG L
	–	C 16562/77	MC	IgG L	IgG L
19	–	C 16800/77	MC	IgG L	IgG L
20	–	C 7460/77	MC	IgG K	IgG L?
21	–	C 8135/77	MC	IgG K	IgG K
	–	233/77	Autopsy	(?) K	IgG K
IgA					
22	Hv	4699/76	MC	IgA L	IgA L
	–	298/76	Autopsy	IgA L	IgA L
23	–	3672/77	MC	IgA K	IgA K
	–	260/77	Autopsy	IgA K	IgA K
24	–	3036/77	MC	IgA K	IgA K
25	–	3771/77	MC	IgA K	IgA K
	–	210/77	Autopsy	IgA K	IgA k
26	–	2074/75	MC	IgA K	IgA K
	–	122/76	Autopsy	IgA K	IgA K
27	Ålb	C 3567/76	MC	IgA K	IgA k
28	–	C 11669/77	MC	IgA K	IgA K
29	–	C 7070/77	MC	IgA K	IgA K
30	–	C 16502/77	MC	IgA K	IgA K
	–	C 582/77	MC	IgA K	IgA K
31	–	C 5407/77	MC	IgA k	IgA K
No M comp					
32	Hv	3169/77	MC	IgD L	No M-comp
33	–	3873/76	MC	IgD L	No M-comp
	–	216/77	Autopsy	IgG L	No M-comp

investigated Thus a total of 52 specimens from 37 cases have been investigated

During the period 1975 to 1977 21 cases were investigated at Hvidovre Hospital by the 2-step technique and 16 cases were investigated by the 3-step technique at Ålborg Sygehus

Tissues

The material was fixed in Lillie's 10% buffered formalin for 6–8 hours After fixation the Radner biopsies were decalcified in 8N formic acid for 6 hours After fixation and decalcification the tissue was dehydrated and embedded in paraffin Serial sections were performed

Antisera

For demonstration of human IgA, IgG, IgM, kappa chains, lambda chains and IgD, rabbit antisera with specific activity against human α, γ, μ kappa chains, lambda chains (DAKO Denmark) and against delta chains (Behringwerke Hoechst, Denmark) were used

The specificity of the antisera was verified by performance testing on sections of formalin fixed and paraffin embedded bone marrow tissue from multiple myeloma of known monoclonal protein type

Working dilutions of antisera were found by titration In the demonstration of heavy chains concentrations from 1/41 to 1/81 were used, in the demonstration of light chains concentrations from 1/81 to 1/201 were used Antisera were diluted in phosphate buffered-saline (PBS) pH 7 2

As a control the immunoglobulin fraction of serum from unimmunized rabbits (DAKO, Denmark) was used diluted 1/81 corresponding to the maximal protein content in the used antisera

In the 2-step IP technique peroxidase conjugated swine-anti-rabbit immunoglobulin (DAKO Denmark) in dilution 1/21 was used

In the 3 step technique swine anti-rabbit immunoglobulin (DAKO Denmark) in dilution 1/41 and complexes of peroxidase anti-peroxidase (PAP) (DAKO Denmark) in dilution 1/101 were used

Staining Procedures

The 2-step IP technique (1) was performed using the following steps after dewaxing the sections

1 Incubation with absolute methanol containing 0 5% H_2O_2 (30 min) in order to block the endogenous peroxidase activity
2 Incubation with 10% normal swine serum (10 min)
3 Incubation with specific rabbit-antiserum in optimal dilutions (30 min)
4 Incubation with peroxidase-conjugated swine anti rabbit immunoglobulin (30 min)
5 Staining with 0 04% 3 amino 9 ethylcarbazol, and 0 01% H_2O_2 dissolved in sodium acetate/acetic acid buffer pH 5 (15 min)

Dilutions were made with PBS containing 2% normal swine serum and rinsing between the steps was also performed in this medium for 15 minutes

After the final rinse the sections were mounted in Aquamount (Gurr)

The 3-step unlabelled IP technique (5) was performed using the following steps

1, 2, 3 As above
4 Incubation with swine anti-rabbit immunoglobulin (30 min)
5 Incubation with PAP (30 min)
6 Incubation with 0 05% 3,3'-diaminobenzidine-tetrahydrochloride (Sigma) and 0 01% H_2O_2 in 0 1 M PBS, pH 7 6 (5 min)

In 3, 4 and 5 antisera and PAP contained 20% normal swine-serum Rinsing between each step was performed in PBS for 30 minutes After the final rinse counterstaining with haematoxylin, dehydration and mounting in Pertex® (Histolab , Bethlehem Trading Ltd) was carried out

In all cases control sections were made in the following manner

a Incubation with the immunoglobulin fraction of serum from unimmunized rabbits instead of specific rabbit antiserum
b Incubation with PBS instead of specific rabbit antiserum

In addition the series of antibodies (anti kappa lambda α, γ and μ) used with each specimen acted as an inherent control by the varying numbers of cells, stained with each antibody

In some cases (v i) the sections were incubated in 0 1% trypsin III (Sigma), 0 1% $CaCl_2$ in distilled water pH adjusted to 7 8 with 0 1 N NaOH for 30 minutes prior to staining (2)

Microscopic Evaluation

Staining reactions were independently evaluated semi quantitatively by 3 of the authors (MJ PJ NT) without any knowledge of the serum M component or other clinical information according to the following scoring system

The number of immunoperoxidase positive cells (0– + + +) the intensity of the staining reaction (0– + + +) in these cells where + + + signified a staining intensity in a normal plasma cell

When the immunoperoxidase reaction for one immunoglobulin (light and heavy chains) or for one light chain alone was positive for a great number of cells (+ + + + +) with an intensity of + + and + + + the picture was said to be monoclonal of this type

Identification of Serum Paraprotein

Qualitative identification of the M component in serum was based on immunoelectrophoresis

RESULTS

The results of the immunoperoxidase staining and serumanalyses are shown in Table 1

In the evaluation of the immunoperoxidase stainings of the tissue total agreement was primarily achieved between the three examiners in 41 out of 52 specimens In furt

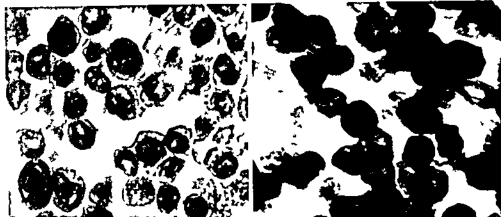


Fig 1 Immunoperoxidase staining of autopsy material from a solitary tumour mass in mediastinum showing a monoclonal staining pattern with tumour cells positive for lambda chains to the right and negative for kappa chains to the left.
(Labelled 2 step IP technique haematoxylin counterstaining oil immersion × 2500 green filter VG 19)



Fig 2 Immunoperoxidase staining of marrow clot from a case with reactive plasmocytosis showing a polyclonal staining pattern with plasma cells positive for lambda chains to the right and for kappa chains to the left
(Unlabelled 3-step IP technique haematoxylin counterstaining oil immersion × 2500)

one or more immunoglobulin fractions but no M component All 3 patients had a clinical diagnosis of rheumatoid arthritis

Comparing the staining reactions of bone biopsies marrow clots and autopsy materials at the same dilution of antiserum the most satisfactory results were found on bone biopsies just as they showed the best morphological preservation Staining of the marrow clots showed somewhat more intense background staining while the autopsy material gave the poorest results The 2 different IP staining techniques showed no difference

DISCUSSION

The demands which should be made on a staining method with reference to its utility in routine diagnostic work are specificity reproducibility and applicability on routinely processed material *Taylor & Mason* (7) and *Pinkus & Said* (4) have proved the specificity of the IP staining with reference to demonstration of intracellular immunoglobulin in patients with multiple myeloma Our results are in accordance with this as we in 44 out of 46 examinations from patients with M-component in

Case no	Histology no	Tissue	IP	Serum or urinary analysis
Light chain disease				
34	Ålb C 8900/77	ICB	L	L
	– C 16501/77	ICB	L	L
Plasmocytosis				
35	Hv 2971/77	MC	K L	IgA SE
			IgA IgG	IgG SE
			IgM	IgM N
36	Hv 7215/76	MC	K L	IgA N
			IgA IgG	IgG N
			IgM	IgM SE
37	Hv 2147/77	ICB	K L	IgA SE
			IgA IgG	IgG N
			IgM	IgM N

Abbreviations used
IP Immunoperoxidase staining
HV Hvidovre Hospital
Ålb Ålborg Sygehus
MC Marrow clot
ICB Iliac crest biopsy
SE Slightly elevated
N Normal

clots 4 autopsies) in which the first examination was inconclusive due to pronounced staining of the background caused by immunoglobulin containing serum repeated stainings with antisera in lower concentrations gave conclusive results

Previous investigations (2) have shown reduced background staining by treatment of the sections with proteolytic enzymes prior to immunohistochemical staining In the ten inconclusive cases the sections were treated with trypsin prior to staining The treatment caused enhancement of both intra and extracellular immunoglobulin staining in bone marrow clots In autopsy material the non specific staining of the tissue was diminished

In only one specimen agreement could not be obtained in regard to heavy chain type in spite of repeated stainings (pt no 21)

In 27 patients with multiple myeloma information about the type of monoclonal heavy and light chains in serum was available Comparison of the results of the IP staining with the serum paraprotein analysis showed total agreement in 25 out of these patients

In 2 patients discrepancies were found In one case repeated serum analyses led to a result corresponding to the IP staining In the other case a second serum analysis has not been performed

In 4 patients (6 specimens) the serum analyses were incomplete as information concerning type of either heavy or light chains were lacking However no discrepancies were found in comparing with the IP stainings

In 1 patient the IP staining as well as serum analysis revealed only monoclonal protein of light chain type

2 patients (3 specimens) had no demonstrable M component in serum In one case immunofluorescence examination on bone marrow smears had shown monoclonal intracellular IgD L and this was confirmed by IP staining on sections from the bone marrow In the other case the patient had a large tumour mass in the mediastinum and the sternum but otherwise no osteolytic changes Post mortem examination with IP staining on sections from the tumour mass showed monoclonal intracellular immunoglobulin of the type IgG L (Fig 1)

In 3 patients with an increased amount of plasma cells in the bone marrow IP staining showed a clear polyclonal immunoglobulin staining pattern (Fig 2) Serum analysis showed a moderate elevation of

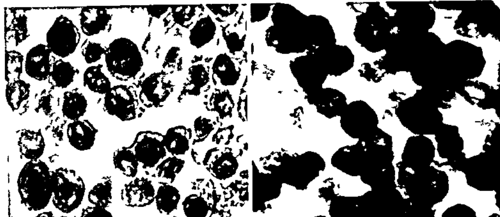


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Fig 2 Immunoperoxidase staining of marrow clot from a case with reactive plasmocytosis showing a polyclonal staining pattern with plasma cells positive for lambda chains to the right and for kappa chains to the left.
(Unlabelled 3 step IP technique haematoxylin counterstaining oil immersion × 2500)

one or more immunoglobulin fractions but no M component All 3 patients had a clinical diagnosis of rheumatoid arthritis

Comparing the staining reactions of bone biopsies marrow clots and autopsy materials at the same dilution of antiserum the most satisfactory results were found on bone biopsies just as they showed the best morphological preservation Staining of the marrow clots showed somewhat more intense background staining while the autopsy material gave the poorest results The 2 different IP staining techniques showed no difference

DISCUSSION

The demands which should be made on a staining method with reference to its utility in routine d agnost c work are spec f~

specificity of the IP staining with reference to demonstration of intracellular immunoglobulin in patients with multiple myeloma Our results are in accordance with this as we in 44 out of 46 examinations from patients with M-component in

serum found total agreement between serum analysis and IP staining

In one case no conclusive result was obtained by IP *staining for heavy chains on autopsy material*

In one case discrepancy between IP staining and serum analysis for light chains was found The serum analysis has however not been repeated and therefore not verified

2 patients had no demonstrable M component in serum but showed monoclonal immunoglobulin production by the IP staining Both cases were probably *non secretory myelomas* (3) *Thus especi*ally in cases with solitary tumour masses without widespread osteolytic changes and with no M component in serum the demonstration of mono clonal immunoglobulin production by IP staining is a valuable diagnostic help

We have in this investigation found a clearly discernible difference between the monoclonal immunoglobulin staining pattern in myelomas and the polyclonal pattern in cases with reactive plasmacytosis Thus when evaluating cases with suspiciously high amounts of plasma cells in the bone marrow the IP staining will be of considerable help

The reproducibility of the IP staining has been proved to be acceptably good With optimal dilution of the specific antisera the evaluations of the three independent examiners were conclusive and concor dant in 51 out of 52 examinations

Repeated dilution of antisera was necessary especially when staining marrow clots and autopsy material in order to obtain the greatest staining contrast between intracellular and extracellular reaction

Trypsination had no effect on the contrast between intracellular and extracellular immunoglo bulin staining of bone marrow material while non specific background staining in autopsy material was reduced

Pretreatment with trypsin seems thus most suitable in staining autopsy material outside the bone marrow as for instance liver and kidney tissue

In agreement with previous findings (4 7) *decalcification* of bone biopsies did not interfere with the demonstration of intracellular immunoglo bulin Furthermore the staining result and morpho logical preservation were better in the biopsies probably due to a more rapid fixation of the smaller specimens Application of the 2 step labelled IP technique and 3 step unlabelled technique has given similar results which speak for the use of the simpler faster and cheaper 2 step method

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We gratefully acknowledge the department of haemato logy and clinical chemistry of Hvidovre Hospital and *Ålborg Sygehus Syd* for their permission to use the *clinical data and the results of serumanalyses*

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FORMATION OF GERMINAL CENTRES DURING NEONATAL TOLERANCE IN THE CHICKEN

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Vainio O & Viljanen M K Formation of germinal centres during neonatal tolerance in the chicken Acta path microbiol scand Sect C *87* 313–317 1979

Chickens were made tolerant to BSA at the time of hatching During the tolerance antigenic stimulation with BSA resulted in poor germinal centre formation as compared to normal immunized control birds The tolerance persisted for at least 6 weeks Its breakdown had hardly started at the age of 12 weeks both IgM and IgG antibody responses against BSA remaining negligible Stimulation with the unrelated antigens SRBC and *Brucella abortus* resulted in good antibody responses but the number of germinal centres was smaller in the tolerant birds than in normal controls

Key words germinal centre neonatal tolerance

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Accepted as submitted 30 iii 79

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... generation of memory cells or to the induction of tolerance Many observations suggest that they may be involved in the generation of memory cells (4 7 12 16) It has been demonstrated that T and B cell interaction is required for both germinal formation and for generation of *memory* (12 13) *Klaus* has produced evidence that antigen antibody complexes together with the third component of complement localize ...
(7) H...
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cells (... *& Parish* propose that reaction of lymphocytes with antigen within the germinal centre might actually result in tolerance (1) However *Cohen & Thorbecke* were not able to demonstrate secondary nodules after stimulating neonatally *tolerant* rabbits with the tolerogen (2) Our own results indicate that germinal centre formation is also influenced by other factors e g the age of the animal and that it is not necessarily directly related to antibody production (14)

The purpose of the present work was to study the formation of germinal centres in the presence or absence of an immune response using chickens rendered tolerant at hatching as an experimental model The results obtained indicate that during the tolerance germinal centre formation as response to stimulation with the tolerogen is reduced greatly

MATERIALS AND METHODS

Experimental design Chickens were rendered tolerant to BSA (bovine serum albumin) at hatching Groups of tolerant birds were immunized with BSA at 1 3 6 and 12 weeks of age Ten days after the immunization the resulting anti BSA antibodies and germinal centres were quantified As controls tolerant non immunized and normal non immunized or immunized birds were studied

Chickens White Leghorn chickens of line V (genotype $B^{15}B^{15}$) from our own colonies were used throughout the study

Tolerance induction Tolerance to BSA was induced in newlyhatched chickens by injecting a single dose of 1250 mg/kg body weight intraperitoneally

Antigens and antigen stimulations BSA (fraction V from bovine plasma Armour Pharmaceutical Company LTD Eastbourne England) was used as antigen Tolerant chicks were divided into four groups and immunized with BSA as follows first group at one week of age with 0 5 mg second group at 3 weeks also with 0 5 mg third group at 6 weeks with 2 5 mg and fourth group at 12 weeks also with 2 5 mg BSA per bird intraperitoneally

As unrelated antigens sheep red blood cells (SRBC) in a dose of 2 × 10⁹ SRBC per bird and phenol killed *Brucella abortus* in a dose of 6 4 × 10⁹ organisms per bird were used and administered intraperitoneally

Antibody assays Blood samples for antibody determination were collected into heparinized syringes ten days after immunization IgM and IgG anti BSA antibodies were measured by enzyme linked immunosorbent assay (ELISA a modification of our RIA method 17) and the antibody concentrations are given as percentage of the same standard plasma in each experimental group Anti SRBC and anti *Brucella* antibodies were titrated as described previously (14)

Autopsy and microscopical examination Ten days after the immunization the birds were killed with ether The preparation of the spleen samples was as described previously (15) On microscopical examination the number of germinal centres in three cross sections was recorded The result was given as the arithmetic mean of the three Each sample was studied without knowledge of its origin

Statistics Student s *t* test was used for the statistical analysis

RESULTS

In order to test the persistence of BSA induced tolerance tolerant chicks were immunized after various time intervals with BSA The IgM and IgG anti BSA antibody production in immunized BSA tolerant and normal chickens and in their non immunized hatchmates is shown in Table 1 In normal non immunized controls the IgM anti BSA antibody level at hatching was 2 8 ± 1 8 per cent of standard plasma whereas no IgG anti BSA antibodies were detected at hatching The control values remained essentially the same throughout the whole experimental period both in neonatally BSA tolerated and in normal non immunized groups Chickens rendered tolerant to BSA at hatching produced only negligible amounts of IgM and IgG anti BSA antibodies following immunization even at 12 weeks of age This finding is in accordance with the observation of *Salerno et al* (10) Our results show that neonatally induced tolerance lasts for at least more than 6 weeks and still at 12 weeks of age the breakdown of the tolerance has scarcely begun

Germinal centre formation in the spleen of tolerant and normal chickens is shown in Table 2 Young birds (1 week old when immunized) formed only one to three germinal centres per cross section of the spleen This was irrespective of whether the birds were tolerant or not Normal immunized

TABLE 1 *IgM and IgG anti BSA Antibody Response in Neonatally BSA tolerated Chickens and Their Controls Mean Values as Percentage of Standard Plasma ± SD are Given*

Chickens	Age at immunization			
	1 wk	3 wk	6 wk	12 wk
IgM anti BSA				
Tolerant BSA immunized	3 7 ± 4 1 (17)	0 5 ± 0 4 (17)	1 4 ± 1 0 (28)	14 7 ± 11 6 (21)
Normal BSA immunized	*11 4 ± 17 9 (15)*	*6 9 ± 8 5 (17)*	*13 1 ± 9 2 (14)*	*117 1 ± 279 7 (12)*
Tolerant non immunized	2 9 ± 3 4 (15)	2 1 ± 2 6 (18)	5 0 ± 7 0 (16)	4 9 ± 4 1 (8)
Normal non immunized	4 4 ± 4 0 (13)	3 9 ± 7 2 (13)	1 5 ± 0 6 (9)	6 3 ± 7 4 (12)
IgG anti BSA				
Tolerant BSA immunized	*0 05 ± 0 1 (17)*	*0 (17)*	*0 4 ± 0 8 (28)*	*7 6 ± 12 4 (21)*
Normal BSA immunized	1 1 ± 2 6 (15)	14 1 ± ?3 6 (17)	22 4 ± 40 0 (14)	101 6 ± 256 6 (12)
Tolerant non immunized	0 (15)	0 (18)	0 (16)	0 05 ± 0 1 (8)
Normal non immunized	0 (13)	0 2 ± 0 2 (13)	0 (9)	0 (12)

Tolerance was induced at hatching by injecting a single dose of 1250 mg BSA/kg body weight intraperitoneally The birds immunized at the age of 1 or 3 weeks were given 0 5 mg BSA intraperitoneally and those immunized at 6 or 12 weeks 2 5 mg Blood samples for antibody assay were taken ten days after immunization Figures in brackets refer to the number of chickens in each group

TABLE 2 *Number of Germinal Centres in the Spleen of Neonatally BSA tolerated Chickens and Their Controls*

Treatment	Age at immunization			
	1 wk	3 wk	6 wk	12 wk
Tolerant BSA immunized	1 6 ± 1 7[a] (17)	5 9 ± 3 8[b] (17)	5 7 ± 3 1 (28)	3 1 ± 3 5 (21)
Normal BSA immunized	2 3 ± 2 1 (15)	16 2 ± 10 3 (17)	6 6 ± 3 6 (14)	1 6 ± 1 5 (12)
Tolerant non immunized	1 2 ± 1 5 (15)	4 9 ± 4 3 (18)	3 4 ± 2 6 (16)	1 8 ± 1 7 (8)
Normal non immunized	0 9 ± 1 1 (13)	6 7 ± 7 0 (13)	7 1 ± 4 0 (9)	1 3 ± 1 2 (12)

Tolerance was induced at hatching by injecting a single dose of 1250 mg BSA/kg body weight intraperitoneally Immunization was carried out at 1 and 3 weeks with 0 5 mg BSA and at 6 and 12 weeks with 2 5 mg BSA intraperitoneally Spleen was removed ten days after the immunization Figures in brackets refer to the number of chickens in each group

[illegible] All other differences between the [illegible]

chickens developed on the average 16 2 germinal centres per cross section at 3 weeks of age whereas neonatally BSA tolerated chickens formed only 5 9 germinal centres per cross section at that age this being roughly the same as in non immunized hatchmates This difference in the germinal centre formation between tolerant and normal birds is statistically significant ($p < 0\ 001$) It is worthwhile to notice that at that age the neonatally tolerated birds produced neither IgM nor IgG anti BSA antibodies At 6 weeks of age no difference in the number of germinal centres was found between tolerant and normal chickens At that age the capacity of normal chickens to form germinal centres is beginning to decrease sharply (14) and this may be one reason why no difference was

TABLE 3 *Anti SRBC and Anti* Brucella *Titres and Number of Germinal Centres in the Spleen of Chickens Rendered Neonatally Tolerant to BSA and Immun[illegible]ed after Various Time Intervals with SRBC and* Brucella

Chickens	Age at immunization			
	1 wk	3 wk	6 wk	12 wk
Anti SRBC titres[a]				
BSA tolerant	2 5 ± 2 1 (10)	3 6 ± 1 1 (10)	3 4 ± 2 7 (9)	5 7 ± 1 8 (9)
Normal	3 5 ± 3 3 (6)	3 1 ± 1 8 (7)	4 6 ± 3 2 (7)	6 0 ± 2 7 (4)
Anti Brucella *titres*[a]				
BSA tolerant	0 2 ± 0 6 (10)	3 5 ± 2 9 (10)	5 0 ± 2 9 (9)	8 0 ± 1 3 (9)
Normal	0 (6)	4 2 ± 2 6 (7)	6 6 ± 2 2 (7)	8 5 ± 1 3 (4)
Germinal centres[b]				
BSA tolerant	5 1 ± 4 8 (10)	5 2 ± 2 0 (10)	4 8 ± 4 9 (9)	3 1 ± 4 6 (9)
Normal	6 7 ± 5 1 (6)	10 0 ± 9 1 (7)	5 1 ± 3 4 (7)	1 0 ± 0 0 (4)

Tolerance was induced as described in previous tables Immunization was carried out at 1 3 6 and 12 [illegible]
× 10^9 SRBC and 6 4 × 10^9 [illegible]
determ[illegible]
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BS[illegible]
[a] M[illegible] [illegible]erences between
[b] A[illegible] mean for three cross sections ± SD

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found Twelve-weeks-old chickens formed only a few germinal centres in accordance with earlier observations (15)

We also tested the influence of BSA-tolerance on antibody production and germinal centre formation after stimulation with the unrelated antigens SRBC and *Brucella abortus* The anti SRBC and anti-*Brucella* titres and numbers of germinal centres per cross section of the spleen are shown in Table 3 The antibody formation of both tolerant and normal control birds was of the same magnitude At the age of 3 weeks the tolerant birds had an average of 5 2 germinal centres per cross section This value is lower than that of normal control birds, viz 10 0 germinal centres per cross section However, the difference is not statistically significant, since two normal control birds showed especially many germinal centres in the spleen

Altogether germinal centre formation during the tolerance was significantly decreased after antigenic stimulation with the tolerated antigen Also after stimulation with unrelated antigens the germinal centre formation in tolerant birds was poorer than in normal controls but in this case the difference was not statistically significant

DISCUSSION

The purpose of the present work was to elucidate the role of germinal centres in the immune response by studying their formation during neonatal tolerance Tolerance was induced according to the protocol described by *Eardley & Tempelis* (3) During the tolerance they observed non-specific immunosuppression (8 9) Birds rendered neonatally tolerant to BSA gave a suppressed response to SRBC stimulus as compared to normal controls In our experiments the tolerance was specific to BSA and stimulation with SRBC and *Brucella* led to normal antibody responses The difference between those findings and our results may be partly explained by different assay methods i e plaque assay versus antibody titration and by differences in the time scale of the experiments *Tempelis et al* observed the strongest non specific suppression when chickens were immunized at two weeks of age and the plaque assay was performed four days later (9) whereas we immunized tolerant chicks at 1 or 3 weeks of age and collected the blood samples for antibody titration 10 days after It is noteworthy however that the tolerant birds formed germinal centres less vigorously than normal control birds after antigenic stimulation with SRBC and *Brucella* The difference was not statistically significant because of the great deviation in the control group

The results of *Ada & Parish* suggest that germinal centres might be related to tolerance induction (1) If this were the case one would expect that increased numbers of germinal centres during early phases and persistence of tolerance could be observed In contrast, we observed a greatly reduced number of germinal centres during tolerance It has been postulated that during the process of germinal centre formation, antigen antibody complexes are carried to lymphoid follicles where they are bound to dendritic reticular cells Lymphocytes are then aggregated around these antigen bearing dendritic cells to form a germinal centre (19) Poor antigen localization has been demonstrated in tolerant animals (6, 18) *Cohen & Thorbecke* have shown that the tolerogen induces reduced germinal centre formation in rabbits rendered neonatally tolerant (2) Our findings are in agreement with these observations At 3 weeks of age tolerant chicks formed very few germinal centres It has been shown that at that age normal chickens form germinal centres vigorously After 6–7 weeks of age, the number of germinal centres after antigenic stimulation begins to decrease evenly parallel with the involution of the bursa of Fabricius (14) Because of the low number of germinal centres detected in the two oldest groups, we could not find any difference between the tolerant and normal birds

When seeking to elucidate the biological role of the germinal centres the following facts should be borne in mind normally the first germinal centres start to appear in chicks about 10 days after hatching, but antigenic stimulation results in an earlier appearance (16) The most vigorous germinal centre formation occurs around 3–6 weeks of age Old birds form germinal centres poorly even after antigenic stimulation and in spite of good antibody responses (5 15) The formation of germinal centres is obviously a complex reaction since histocompatible T and B cells are required in addition to dendritic reticular cells (13) The present results demonstrate in accordance with earlier observations in rabbits (2) that during the tolerance germinal centre formation as response to the tolerogen is poor Possibly studies with isolated germinal centres (11) may prove helpful in elucidating their immunological function

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AUTOANTIBODIES IN SERUM AND SPUTUM FROM PATIENTS WITH CYSTIC FIBROSIS

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Schiøtz P O Egeskjold E M Høiby N & Permin H Autoantibodies in serum and sputum from patients with cystic fibrosis Acta path microbiol scand Sect C 87 319–324 1979

Sera from 89 patients with cystic fibrosis (CF) and 88 control persons were examined for the occurrence of rheumatoid factors (RF) of the IgG IgA and IgM classes by an indirect immunofluore scence method and by the latex fixation slide test The prevalence of RF IgG was significantly higher (88%) ($p<0.0005$) among the CF patients than among the control persons (7%) while no difference was found between the two groups with regard to RF of the IgA or IgM classes Fifty five of the CF patients had chronic *Pseudomonas aeruginosa* infection in their lungs and two or more precipitins against these bacteria in their sera determined by crossed immunoelectrophoresis These CF patients did not differ from the 34 CF patients without chronic *P aeruginosa* infection neither with regard to prevalence nor titer of RFs but there was a positive correlation between the number of *P aeruginosa* precipitins in the 55 chronically infected CF patients and their titers of IgG RF Nineteen CF patients were examined also for RFs antinuclear antibodies (ANA) and anti DNA antibodies in their sputum sol phase and corresponding sera RFs were demonstrated in the sputum sol phase from 6 of the patients by the latex fixation test, whereas their sera were negative in this test possibly indicating a local production of RF Positive reactions for ANA and anti DNA antibodies were found in 7 and 10 of the sputa respectively and in higher titers than in the corresponding sera also suggesting a local production Titers of autoantibodies in sputum were low and no difference was found between patients with chronic *P aeruginosa* infection and patients without *P aeruginosa* infection The possible role of autoantibodies in the patogenesis of pulmonary tissue damage in CF patients is discussed

Key words Rheumatoid factors antinuclear antibodies anti DNA antibodies cystic fibrosis

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Autoantibodies in serum in the form of rheuma toid factors (RF) and antinuclear antibodies (ANA) have been demonstrated in a variety of infectious diseases with a prolonged or chronic course These autoantibodies seem to be associated with cell degradation hyperimmunization against the causa tive agent and immune complex formation between microbial antigens and the corresponding antibodies (1 2 4 10 33 34)

In patients with cystic fibrosis (CF) who suffer from recurrent and chronic infections – especially with *P aeruginosa* (12) one finds pronounced cell degradation as well as hyperimmunization against *P aeruginosa* (13) and immune complexes presu mably between *P aeruginosa* antigens and the corresponding antibodies have been demonstrated also (28 29) In previous studies ANA was found *in serum* from 1/4 of these patients but the prevalence of RF of the IgM class – as determined

Behring Werke (West Germany). Monospecificity was secured as described for ANA. To avoid non-specific staining the antisera were absorbed with Rabbit Liver Powder (Difco) 100 mg/ml. All sera and sputa were tested by the Latex fixation test (Behring-Werke test kit) (26).

Antinuclear Antibodies and Anti DNA Antibodies

Investigations for the occurrence of granulocyte specific (GS) and organ non specific (ON) ANA belonging to the IgG, IgA and IgM classes were also carried out. Rat liver cryostat sections and smears of isolated and washed human leucocytes served as nuclear substrates (35, 36). All the sputa and their corresponding sera were screened for IgG, IgA and IgM ANA undiluted and diluted 1:4. This latter dilution was used to avoid false negative reactions due to prozone phenomena. Positive samples were semiquantified by twofold dilutions using PBS pH 7.2 as diluent. The sputa and corresponding sera were furthermore investigated for the occurrence of anti DNA antibodies of IgG, IgA and IgM classes and the kinetoplast of *Crithidia luciliae* served as DNA substrate (25). The detailed immunofluorescence technique has been described earlier (25).

Conjugates. FITC-labelled rabbit IgG specific for human α, γ and μ chains were from Dakopatts (Copenhagen). The anti immunoglobulin conjugates were tested for specificity by means of IgG, IgA and IgM monoclonal bone marrow specimens from patients with multiple myeloma and macroglobulinaemia (35). All antisera showed monospecific reactions in crossed immunoelectrophoresis. The fluorescein/protein ratios as estimated by OD 495/280 nm were 0.5–0.8. The slides were examined in a Leitz Orthoplan fluorescence microscope equipped for incident light illumination (28).

Statistical Methods

The Mann Whitney test, Fisher's test, Spearman's correlation coefficient R and the X^2-test were used and a significance level of 5% (double tailed test) was chosen (7).

RESULTS

In Table 1 the distribution of the different titers within each RF class is given for serum from the CF patients. In the control group only 6 persons (7%) were positive for IgG RF and 3 (3%) were positive for IgM RF – all in lowest titer – while none were positive for IgA RF. The results demonstrate the occurrence of RF IgG to be significantly higher among CF patients than in the control group ($X^2 = 116$, d.f. 1, $p < 0.0005$). No significant difference in occurrence of IgA RF or IgM RF was found between the two groups. All patients except one (1%) had a negative RA latex fixation test in serum.

In 55 CF patients 2 or more *P. aeruginosa* precipitins were found in serum. This has previously been demonstrated to indicate a systemic humoral immune response to *P. aeruginosa* infection (14, 15) and high numbers of *P. aeruginosa* precipitins are known to be closely correlated to a poor prognosis (16). The results showed that there were no significant differences between CF+P and CF–P with regard to the prevalence or titer of RF IgG in serum ($p > 0.05$). There was, however, a positive correlation between the number of *P. aeruginosa* precipitins and the titer of IgG RF in the CF+P group (Spearman's rho = 0.445, $p < 0.01$).

The latex fixation test was positive in the sputum sol phase from 6 out of 19 patients (32%) in titers from 1:4 to 1:16. The corresponding sera were examined undiluted and in dilution 1:4 as well as 1:40 and all were negative thus indicating a possible local production of RF. Four of these patients were from the CF+P group and the remaining two patients were from the CF–P group.

Examination for RF of IgG, IgA and IgM class by the immunofluorescence technique in sputum sol phase was negative for both the CF+P and the CF–P patients. With regard to ON ANA and anti DNA antibodies in sputum the results are given in Table 2. The titers of ANA in sera corresponding to the positive sputa were in most of these cases lower indicating a possible local production of ON-ANA of IgG, IgA and IgM classes in one, five, and one patient respectively. Thus a total of seven patients out of (37%) had possibly a local ANA production while GS-ANA was not demonstrated in the sputum.

The titers of the anti DNA antibodies in sera corresponding to the positive sputa were in most

TABLE 1. *Occurrence and Titers of Rheumatoid Factors (RF) og IgG, IgA and IgM Classes in Serum of Cystic Fibrosis Patients*

Titer	0	1:9	1:18	1:36	Pos./Total
IgG RF	11 (12%)	12 (13%)	30 (34%)	36 (40%)	78/89 (88%)
IgA RF	87 (98%)	2 (2%)	0	0	2/89 (2%)
IgM RF	81 (91%)	4 (5%)	3 (3%)	1 (1%)	8/89 (9%)

by the conventional RA latex fixation test – was only 6% (14 28)

The aim of the present study has been to investigate whether RFs of other Ig classes than IgM can be found in serum of CF patients with special regard to patients suffering from chronic *P aeruginosa* infection (16) We furthermore wanted to examine whether RF and ANA autoantibodies were present in the sputum sol phase from CF patients

PATIENTS AND METHODS

Patients

Sera from 89 CF patients were studied (54 males and 35 females) with an average age of 12 years (range 2–30 years) They all had a typical history of CF and markedly elevated sweat electrolytes determined by the pilocarpine iontophoresis method (9) All patients have been followed every month in our clinic with bacteriological examination of sputum or tracheal aspirations and evaluation of clinical status as detailed previously (11 12) Furthermore in 19 of the abovementioned CF patients the sputum sol phase was examined also

The patient groups consisted of (i) 11 patients (6 males 5 females, mean age 15 years range 7–26 years) suffering from chronic infection with mucoid *P aeruginosa* (CF+P) and each having more than 10 different *P aeruginosa* precipitins in their serum (13) The mean duration of the *P aeruginosa* respiratory tract infection was 4 years (range ½–7 years) Routine studies of the lung function showed a reduced vital capacity and peak expiratory flow rate as compared with normal values (32)

(ii) 8 CF patients (6 males 2 females mean age 15 years range 8–21) without *P aeruginosa* infection (CF–P) and with 0–1 *P aeruginosa* precipitins were examined also The average vital capacity of their lungs was –1 SD below mean normal values and the peak expiratory flow rate was + 0 3 SD above mean normal values (32) In 5 of the CF–P patients *S aureus* or *H influenzae* were isolated from the respiratory tract during this study Sera from a control group of 88 persons (44 males and 44 females) were examined also Their average age was 12 years (range 2–30 years) The control group consisted of 64 children without any signs of infection – who were admitted to hospital for minor surgical operations and 24 healthy blood donors

odies was investigated by means of crossed immunoelectrop resis (microtechnique) as described previously (13) A close correlation between the presence of precipitating anti bodies in serum and sputum sol phase has been observed (27)

Sputum

A 3 h sample of sputum was collected at 4°C (to inhibit proteolytic activity) from each patient between 8 a m and 11 a m as described previously (3 27) Each sample was examined bacteriologically by microscopy and culture and the origin of the specimens from the lower respiratory tract was confirmed by the presence of respiratory epithelial cells and the absence of a significant number of squamous epithelial cells (12 27) Sol phase of sputum was obtained by ultracentrifugation at 120000 × *g* (maximum value) at 4°C for 4h in an SW 50 1 rotor model L2 65 B Beckmann preparative ultracentrifuge as detailed previously (27) and the sol phase was subsequently stored in small aliquots at – 80°C

Rheumatoid Factor Examination

The sera and sputa were inactivated at 56°C for 30 minutes before examination for RF of the IgG, IgA and IgM classes The method employed is a modification of the method of *Estes* (8) It is specific for RF – IgG IgA and IgM respectively (17)

Briefly a 30% suspension of formalin fixed erythro cytes in 0 9% NaCl is smeared on glass slides and sensitized with rabbit anti sheep erythrocyte 7S globulin (IgG) (amboceptor) On the slides are then layered serial dilutions of patients sera The slides are incubated for 30 minutes at room temperature in a moist chamber and

(working dilution 1 10) and after incubation for 30 minutes and washing in PBS the slides are mounted in PBS glycerol 1 1, and read in a fluorescence microscope (Ortholux Leitz)

BG 38 glass filter, a KF 490 interference filter and a TK 495 dicroic mirror for selection of excitation light while a KP 510 glass filter served as barrier filter

When examining for IgG RF it is necessary to destroy IgM RF which if present may give false positive IgG RF reactions (17) This was done by incubating the patients sera with dithiothreitol (Sigma Chem Co) (DTT) 0 154 g/100 ml 1/15 M PBS pH = 7 4 Serum and DTT solution were mixed 1 2 and then incubated at 37°C for 60 minutes and the latex test and the indirect immunofluorescence test for IgM RF were then found to be negative After incubation the mixture was further diluted with PBS to the serum dilutions of 1 9 1 18 and 1 36 before application on the test slide

Control slides Each serum was examined in parallel with a slide omitting the amboceptor This control was in no instance found to be positive Furthermore in each series control slides were included to detect any reaction between the FITC anti IgM or IgG and the sensitizing serum or the sheep red blood cells Finally a known positive and negative serum was included All the slides were read blindly by one of the authors

Amboceptor Rabbit anti sheep 7S globulin was obta ined from Nordic (Tilbury The Netherlands) It was PBS a protein concentration of 2 5 mg/ml

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TABLE 2 *Occurrence of Organ Non Specific Antinuclear Antibodies (ON ANA) and Anti-DNA Antibodies of the IgG, IgA and IgM Classes in Sputum Sol Phase from Cystic Fibrosis (CF) Patients*

19 CF patients	IgG positive/ total	IgA positive/ total	IgM positive/ total
ON ANA	1/19	6/19	4/19
anti-DNA antibodies	7/19	9/19	2/19

cases lower indicating a possible local production of anti-DNA antibodies of IgG, IgA and IgM classes in six, five, and two patients respectively Thus a total of ten patients out of 19 (53%) had possibly a local anti-DNA antibody production (three patients produced anti-DNA antibodies of two immunoglobulin classes)

All of the positive titers for ON-ANA and anti-DNA antibodies in sputum were low (max 1 8) and there was no significant difference in occurrence of these types of antibodies when the CF + P patients were compared with the CF–P patients Only two patients have a possible local production of both ON-ANA and anti-DNA antibodies – in both cases of the IgA class

DISCUSSION

The finding that autoimmune antibodies are frequent in patients with chronic respiratory diseases accompanied by lung infection is confirmed by our results (1, 2, 10,33) The prevalence of IgG RF in serum was as high as 88% There was no significant difference between the 55 patients with *P aeruginosa* precipitins and the 34 CF patients without *P aeruginosa* precipitins neither with regard to prevalence nor to titer of IgG RF This is not surprising since CF patients contract frequent lung infections with many different pathogens (12) The correlation of the IgG RF titers with the titers of *P aeruginosa* precipitins may be explained by increased possibilities for antigen-antibody reactions between *P aeruginosa* antigens and the corresponding IgG antibodies in patients with many *P aeruginosa precipitins* Such reactions may induce IgG RF antibodies against hidden antigenic determinants of the patient's IgG which are exposed during immune complex formation (1, 4, 21, 28, 29) This is interesting since a high number of specific precipitins is closely associated with chronic *P aeruginosa* infection and a poor prognosis (13, 16)

Our inability to demonstrate RFs in the sputum sol phase by the immunofluorescense technique was surprising, compared to our findings with the latex fixation test The considerable proteolytic activity in purulent secretions is well documented (20) and with the necessary heating of the sputum sol phase to 56°C in 30 minutes before examination for RF by immunofluorescence, enzymatic clevage of the RFs may have taken place This is supported by the positive latex fixation tests in six patients (the latex fixation test does not require heating of the specimens) Another explanation might be that the IgG RFs are bound to immune complexes present in the sputum sol phase and thus avoid detection, or the autoantibodies may have been removed as cryoprecipitable immune complexes during the preparation of the sputum sol phase

As far as local ANA and anti-DNA antibody production is concerned we found no difference between the CF + P and the CF–P patients in the present work but 68% of the CF patients possibly have a local production of ANA or anti-DNA antibodies mostly of the IgA class

The high prevalence of autoantibodies in serum and sputum of CF patients is in accordance with the concept that the production of RFs may be a secondary event following repeated immunization with a foreign antigen The recent results of *Carson et al* (4), who examined patients with subacute bacterial endocarditis longitudinally, showed that the high titres of IgG RF demonstrated in these patients fell to normal values after treatment of the underlying bacterial disease (4) The tissue damage caused by the lung infections in CF patients will subsequently stimulate to production of ANA and anti-DNA antibodies Several investigators, however find it unlikely, at least in rheumatoid arthritis that autoantibodies – notably RFs – are merely benign indicators of antigenic stimulation (22) *In* experimental systems it has been shown that RF accentuated pulmonary lesions associated with diffuse proliferative lung disease (6), and deposits of IgM RF have been demonstrated in the lungs in patients with rheumatoid lung disease (5) There is furthermore suggestive evidence for the participation of ANA and RF in the formation of immune complexes (18 19 24 30, 31 37) and ANA and RF may participate in the immune complex activity, which has been demonstrated in sputum from CF + P patients (23, 28 29)

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CYTOSTATIC AND PHAGOCYTIC CAPACITY OF LYMPHOKINE-ACTIVATED HUMAN MONOCYTES

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Unsgaard G Cytostatic and phagocytic capacity of lymphokine activated human monocytes Acta path microbiol scand Sect. C 87 325-332, 1979

Human monocytes were rendered cytostatic to the human cell line NHIK 3025 by exposure to lymphokine supernatants (LS) from BCG stimulated lymphocytes Exposure to LS for 1, 2 and 4 h induced a considerable cytostatic capacity in the monocytes However, a stronger cytostatic effect was acquired by exposure to LS for 24 h and 72 h The phagocytosis of ¹²⁵I labelled *Candida albicans* by *LS* activated monocytes was compared with phagocytosis by monocytes treated with control supernatants (CS) The ingestion was increased by short exposure to LS However a 72 h exposure to *LS* induced a decreased ingestion capacity The capacity of the LS activated monocytes to digest ingested *C albicans* was suppressed DNA synthesis was increased in the LS activated monocytes while protein synthesis was not significantly influenced The cytostatic capacity of LS-activated monocytes was abolished by culture for 24 h after removal of LS Following removal of LS or CS ... of differentiation were less ... also reduced in the former

Key words Monocytes cytostasis phagocytosis, lymphokine activation human

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Previous experiments in our laboratory have demonstrated that cytostatic and cytocidal capacity to the human cell line NHIK 3025 can be induced in human monocytes both by *in vitro* culture in the presence of human serum (6) and by lymphokines (1 7) The former procedure brings about differentiation of the monocytes into large cells (12) showing increased protein synthesis (6) *and increased* phagocytosis of heat killed *Candida albicans* (9) The monocytes had to be cultured ... 9 days ... cells ... stror ... only ... as to whether lymphokines accelerate the differentiation seen ...

... DNA ...

MATERIALS AND METHODS

Cell cultures Mononuclear leucocytes were isolated from venous blood obtained from healthy BCG vaccinated volunteers (8) Monocytes were cultured at the bottom of dishes (Costar 3506 Ø 35 mm) for the cytostatic assay and on coverslips (10 5 × 22 mm) for the phagocytic assay and for the morphological study The coverslips were placed ... non adherent cells removed 90 min later (7) Target cell in the cytostatic ...

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A

Values in cultures exposed to LS as percentage of values in cultures exposed to CS

100 90 80 70 60 50

1/12 1 2 4 24 72

Duration of lymphokine exposure hours

B

Values in cultures exposed to LS as percentage of values in cultures exposed to CS

260 240 220 140 120 100 80 60 40

(±33)

1 2 4 24 72

Duration of lymphokine exposure hours

Fig 1A Cytostatic capacity of monocytes exposed to lymphokines for different intervals of time The graph shows ^{3}H TdR incorporation in co-cultures of LS-exposed monocytes and tumour cells expressed as percentage of ^{3}H TdR incorporation in co-cultures of CS-exposed monocytes and tumour cells n = 6

Fig 1B Phagocytosis of ^{125}I labelled *C albicans* by monocytes exposed to lymphokines for different intervals of time The graphs show total radioactivity (O O) percentage radioactivity released to supernatant (△——△), and percentage radioactivity in the sediment (□ □) for LS-exposed monocytes expressed as percentage of the values for CS-exposed monocytes n = 6

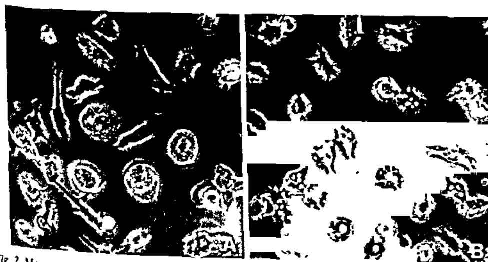


Fig 2 Monocytes exposed to CS (A) or LS (B) from 72nd to 96th h of culture (× 400)

pooled human ABRh + serum, 0 1 mM l-glutamine and 40 μg gentamicin per ml (HS M)

Lymphokine supernatants Lymphocytes were cultured in a concentration of 10^6 cells per ml HS-M in round-bottomed tubes (93 × 24 mm) Lymphokine supernatants (LS) were produced by stimulating the lymphocytes with *Mycobacterium bovis*, strain BCG (Statens Seruminstitut, Copenhagen, Denmark), as described previously (7) Control supernatants (CS) were obtained from non-stimulated lymphocyte cultures After 24 h the supernatants were collected, filtered (Millipore 0 45 μm) and, if necessary, stored at 4°C

Lymphokine activation Monocytes were incubated for various periods of time with LS or CS diluted 1 2 in fresh HS-M The supernatants were then removed and different monocyte functions were tested At that time monocytes had usually been cultured *in vitro* for 4 days

In some experiments the lymphokine activated monocytes were subsequently cultured in HS M or FCS-M (25 per cent foetal calf serum obtained from Gibco, Bio Cult Glasgow, Scotland, substituted for the human serum) for 4 or 24 h before cytostatic ability was tested In other experiments, activated monocytes cultured in HS M for 24 h were re-exposed to lymphokines

Survival of monocytes was registered by counting attached cells in an inverted phase contrast microscope (× 400) Ten visual fields were counted per culture The value per experiment was the mean value of duplicate cultures

Cytostatic capacity of monocytes was registered as described previously (6) Briefly, 10^5 NHIK 3025 cells in 1 ml HS M were added per dish making the monocyte/tumour cell ratio about 2 1 The monocytes had been washed with warm RPMI 1640 prior to addition of tumour cells The monocytes and tumour cells were co cultured for 24 h Four hours prior to termination, 0 5 μCi ^{3}H-thymidine (^{3}H TdR) was added per ml In some experiments ^{3}H TdR was added either in the HS M used from the start of the 24 h assay or in fresh HS M The two procedures gave almost identical results Therefore ^{3}H-TdR was later added without change of medium The results are usually expressed as ^{3}H-TdR incorporation (c p m) in monocyte/tumour cell co cultures calculated as percentage of ^{3}H-TdR incorporation in tumour cells cultured alone

Cytocidal capacity of monocytes NHIK 3025 cells were labelled with ^{3}H TdR (6) 2 × 10^4 labelled cells in 2 ml HS M were added per dish making the monocyte/tumour cell ratio about 10 1 The dishes had been washed with warm RPMI prior to addition of tumour cells The cells were co-cultured for 72 hours The radioactivity released to the supernatants was calculated as percentage of total radioactivity

Phagocytosis For this assay monocytes were cultured on coverslips One ml of a suspension of 2 × 10^6 heat killed, ^{125}I labelled *C albicans* (9) in HS M was added per dish After an incubation period of 15 min the coverslips were washed 12 times in RPMI 1640 and placed in new dishes with fresh HS-M for further incubation The cultures were harvested 24 h later The medium was centrifuged (1800 G for 10 min) and the radioactivity in the supernatants, in the sediments and on the coverslips was measured in a Packard Auto-Gamma counter

Protein synthesis and DNA synthesis in lymphokine-activated monocytes In order to minimize contamination of adherent lymphocytes, the monolayers were washed six times with warm RPMI prior to addition of lymphocyte supernatants One μCi ^{3}H-leucine (sp act 1 0 Ci/mmol) or 0 5 μCi ^{3}H-TdR (sp act 25 Ci/mmol) was added in 1 ml fresh HS-M 4 h prior to harvesting

Harvesting Both in the cytostatic assay and in the assays measuring protein synthesis and DNA synthesis in monocytes the cells were lysed by distilled water and harvested on filters by a Titertek multiple cell harvester (6)

Morphology Cells cultured on coverslips were studied in micro chambers (12)

Statistics All experiments were performed in duplicate Results are given as the mean ± SEM of n experiments The p values were calculated using the Wilcoxon two-sample test

RESULTS

Cytostatic and Cytocidal Capacity of Lymphokine-activated Monocytes

In six experiments, monocytes exposed to lymphokine supernatants (LS) for 72 h inhibited DNA synthesis in tumour cells to a mean of 51 ± 8 per cent of controls (tumour cells alone) Monocytes exposed to control supernatants (CS) did not inhibit DNA synthesis in tumour cells, the mean value being 111 ± 3 per cent of controls The lymphokine activated monocytes also had a strong cytocidal effect on tumour cells, measured as release of radioactivity from prelabelled tumour cells (6) during 72 h of co-culture The spontaneous release from tumour cells was 28 ± 3 per cent, the release from co cultures with CS treated monocytes was 38 ± 8 per cent and from co-cultures with LS-activated monocytes 64 ± 17 per cent (n = 5)

The kinetics of the induction of cytostatic capacity showed that the monocytes had to be exposed to LS for more than 5 min to be rendered cytostatic (Fig 1A) Monocytes exposed to LS for 1, 2 and 4 h were weakly cytostatic, while 24-h exposure induced almost as strong a cytostatic effect as exposure for 72 h

Concomitant with the induction of cytostatic capacity in the monocytes, there were morphological changes (Fig 2B) The number of perinuclear phase-dense granules was reduced and vacuoles appeared in the cytoplasm of the activated monocytes The cells treated with CS showed about the same morphology as cells cultured in HS-M (Fig 2A)

Fig 1A Cytostatic capacity of monocytes exposed to lymphokines for different intervals of time The graph shows ^{3}H TdR incorporation in co-cultures of LS-exposed monocytes and tumour cells expressed as percentage of ^{3}H TdR incorporation in co-cultures of CS-exposed monocytes and tumour cells n = 6

Fig 1B Phagocytosis of ^{125}I labelled *C albicans* by monocytes exposed to lymphokines for different intervals of time The graphs show total radioactivity (O —— O) percentage radioactivity released to supernatant (Δ——Δ) and percentage radioactivity in the sediment (□ —— □) for LS-exposed monocytes expressed as percentage of the values for CS-exposed monocytes n = 6

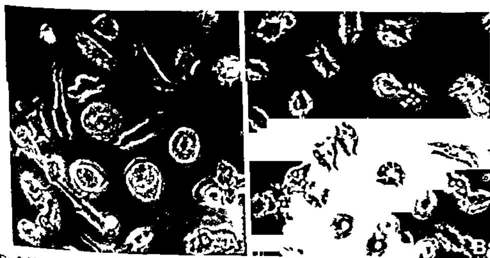
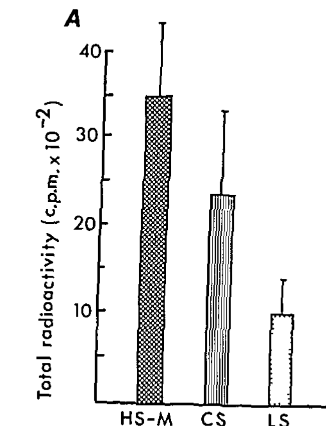
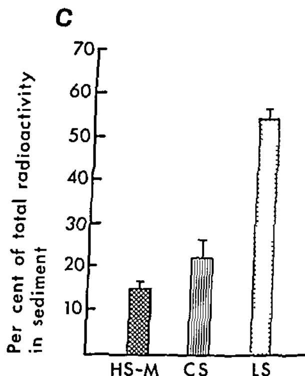


Fig 2 Monocytes exposed to CS (A) or LS (B) from 72nd to 96th h of culture (× 400)





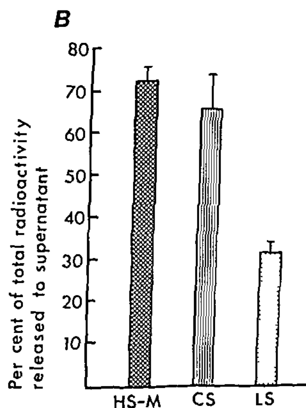


Fig 3 Phagocytosis of ^{125}I-labelled *C albicans* by monocytes cultured for 96 h in HS-M (▩) or exposed to CS (▥) or LS (▭) from 24th to 96th h The columns show total radioactivity (A), and percentage of total radioactivity found in supernatant (B) and in sediment (C) at the termination of the 24-*h* assay $n = 6$

Phagocytic Capacity of Lymphokine-activated Monocytes

Monocytes exposed to LS for 72 h showed decreased capacity to ingest radio-labelled *C albicans* (Fig 3A), as measured by total radioactivity (c p m coverslips + c p m sediment + c p m supernatant) This was not due to alteration in number of cells, since there was no significant difference in the survival of LS activated monocytes as compared to controls treated with CS or incubated in HS-M (Table 1) counted after 4 days of

TABLE 1 *Survival of Monocytes during Lymphokine Activation and during Post-activation Culture in HS-M*

Monocyte exposure from 24th to 96th h in culture	No of cells per visual field	
	Day 4[a]	Day 8[b]
LS[c]	39 ± 4	28 ± 5
CS	38 ± 5	45 ± 8
HS M	38 ± 5	44 ± 6

[a] Termination of the lymphokine activation period $n = 10$

[b] Termination of 4 days culture in HS M after lymphokine activation $n = 10$

[c] LS Lymphokine supernatants CS Control supernatants HS-M

culture The digestion of the ingested particles measured as percentage of total radioactivity relea sed to the supernatant was strongly suppressed in *LS-activated monocytes* (Fig 3B) The radioactivity in the sediment indicating detachment of cells containing und gested particles was strongly increa sed in the LS activated cultures (Fig 3C)

The ingestion capacity of the monocytes exposed to LS for up to 24 h was increased slightly the increment being significant ($p<0\ 05$) for the 1 h exposure only However a signigicantly reduced d gestion capacity ($p<0\ 05$) was noted after LS exposure for 4 h Moreover a significantly increa sed detachment occurred after LS exposure for 2 h ($p<0\ 05$) Thus the lymphokine induced reduction of d gestion capacity and in particular the reduction of ingestion capacity is retarded as compared to the induct on of cytostatic capacity (Fig 1A and 1B)

Effect of Lymphokine Activation on Protein Synthesis and DNA Synthesis in Monocyte Monolayers

The ^{3}H leucine incorporation was not signifi y ($p>0\ 5$) altered in the LS activated monocy s compared to the CS treated monocytes (Fig The ^{3}H TdR incorporation was however increased sixfold in the LS activated cells as compared to the CS treated cells ($p<0\ 01$) Despite thorough washing of the monolayers it might be that the increased DNA synthesis was due to stimulation of adherent lymphocytes However the mitogenic effect of LS on $2\ 5\times10^5$ lymphocytes (almost equalling the number of adherent cells per dish) was tested by using the same activation protocol *The ^{3}H TdR incorporation was found to* increase to a mean value of 410 ± 130 c p m ($n-5$) which is a 60 per cent increase as compared to CS treated lymphocytes Accordingly it seems reasonable to assume that there is an activity in LS which induces DNA synthesis in adherent monocy tes

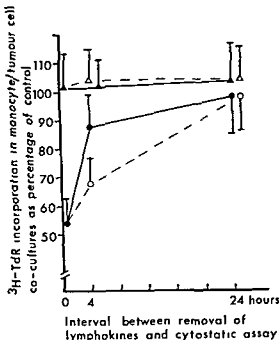
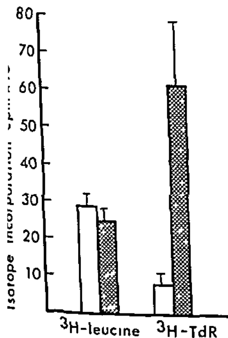


Fig 5 Reversal of cytostatic capacity after removal of LS Monocytes exposed to CS (▲ △) or LS (● O) were tested immediately and after culture for 4 and 24 h in FCS M ( ) or HS M (—) n = 5



g 4 Prote n synthesis and DNA synthesis in onocyte monolayers exposed to LS (▨) or CS (□) ne μCi ^{3}H leucine or 0 5 μCi ^{3}H TdR *respectively* ere added in 1 ml fresh HS-M 4 h prior to harvesting 6

Reversal of Activation after Removal of LS from the Monocytes

The cytostatic capacity of activated monocytes was lost readily after removal of LS Post activation culture of monocytes in HS M for only 4 h before starting the assay brought about a drastic reduction in cytostatic capacity (Fig 5) Post activation culture *in FCS M caused a slower reversal of the activation* After 24 h the monocytes had lost the LS induced cytostatic capacity

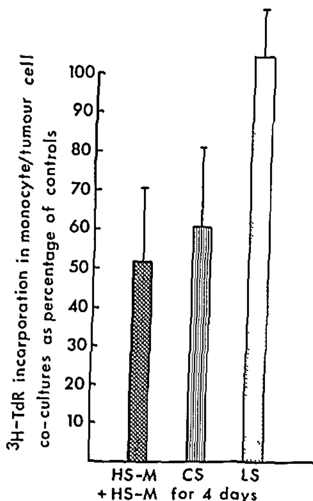


Fig 6 Cytostatic capacity of monocytes cultured from 24th to 96th h in HS-M (▨) CS (▥) or LS (□), and then for 4 days in HS M n = 5

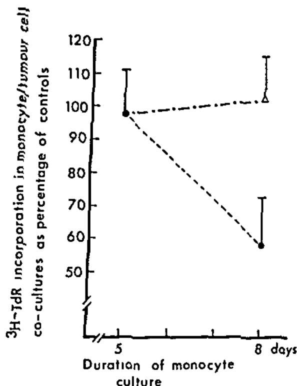


Fig 8 Reactivation of monocytes LS-activated monocytes were cultured for 24 h in HS-M and then the cells were reexposed to CS (●- - -△) or LS (●- - - -●) for 72 h before the cytostatic capacity was tested n = 6

Reduced Differentiation during Post-activation Culture in HS-M

Development of cytostatic monocytes by prolonged *in vitro* culture in HS-M appeared to be blocked by previous LS-activation (Fig 6) This could be due to reduction in the monocyte/tumour cell ratio, since there was a decreased survival of monocytes in the post-activation period, measured as the number of cells 4 days after removal of LS (Table 1) By matching CS- and LS-exposed monocyte cultures containing about the same number of cells, it was found that the mean cytostatic capacity in four such pairs was 55 ± 13 per cent of controls for previously CS-exposed monocytes (cell number 30 ± 6 per visual field)



Fig 7 Monocytes exposed to CS (A) and LS (B) from 72nd to 96th h and subsequently cultured in HS M for 4 days (× 400)

and 101 ± 9 per cent of controls for monocytes previously activated with LS (cell number 30 ± 6 per visual field) Monocytes activated by a 24 h exposure to LS also showed reduced survival and reduced development of cytostatic ability during subsequent culture in HS M

The morphology of LS activated monocytes cultured subsequently in HS M for 4 days was different from that of monocytes treated previously with CS (Fig 7A and 7B) The activated monocytes were less dispersed and showed less perinuclear phase-dense granules than CS treated monocytes

Reactivation of Monocytes

activated the cytostatic ability of these cells (Fig 8)

DISCUSSION

The results show that lymphokine activation affects monocyte physiology in a way unlike the differentiation brought about by culture in the presence of human serum While the cytostatic capacity induced by prolonged *in vitro* culture in human serum was accompanied by *increased* phagocytic capacity and increased protein synthesis (6) the lymphokine activation of the cytostatic capacity was accompanied by *depressed* phagocytic capacity and no alteration in protein synthesis The cytostatic capacity of activated monocytes was readily reversed by culture in the presence of human serum after removal of lymphokine supernatants This reversal was slower in the presence of foetal calf serum Considering that human serum is a better inducer of differentiation this supports the idea of lymphokine activation being brought about by a mechanism which is different from the mechanism of differentiation. However there are other explanations for the slower reversal of activation in foetal calf serum Recently endotoxin has been detected in commercial sera (10) and endotoxin increases the lymphokine activation (3)

One explanation for the results presented in this article could be that LS *contain* lymphotoxines which disintegrate monocytes thereby releasing material toxic to tumour cells However this interpretation is unlikely for several reasons Firstly survival of monocytes following lymphokine activation is not *reduced* as compared to controls Secondly protein synthesis is not reduced in the activated monocytes Thirdly DNA synthesis in the monocytes is increased

Another explanation could be that cytostatic ability and phagocytic ability are dependent on entirely different mechanisms Phagocytosis of heat killed *C albicans* is due to membrane functions (9) and probably to activity of lysosomal enzymes (11) Cytostatic ability could be due to some other mechanism such as the hydrogen peroxide/superoxide system or another as yet unknown system Because of energy economy lymphokines would then increase the cytostatic function on behalf of the phagocytic function Several observation are in favour of this hypothesis Firstly the reduced differentiation in the post activation period is in agreement with commitment of monocytes by lymphokines Secondly the strong reactivation of the monocytes might indicate a metabolic reorganization during the first activation Thirdly the increased DNA synthesis indicates an active process Fourthly the kinetics of lymphokine activation show a rapid onset of the cytostatic effect while in comparison the depression of digestion is somewhat delayed and the depression of ingestion much delayed A change in the metabolism from the maintenance of phagocytic functions would lead to a gradual depression because of the turnover of the substances (enzymes cytoplasmic membrane) involved in phagocytosis

A third explanation might be that lymphokines induce a de stabilization of membranes with release of intracellular material to the extracellular milieu including enzymes necessary for digestion of *C albicans* This could be due to a physiological mechanism or weak injury to the monocytes by toxic substances The increased detachment of monocytes during the phagocytic assay indicates a toxic effect though a mobilization of cytoplasmic membranes for other functions than attachment would also lead to increased detachment The reduced differentiation and the reduced survival during post activation culture could be due to a toxic effect of lymphokines However the strong reactivation opposes this explanation

Neither the hypothesis indicating a lymphokine induced alteration in metabolism nor the hypothesis indicating a lymphokine induced release of intracellular material is finally confirmed by the results

results which support this observation *Nathan & Terry* (4) found that peritoneal macrophages from BCG infected mice show decreased *phagocytosis* (ingestion) as compared to macrophages from untreated mice Macrophages from BCG treated

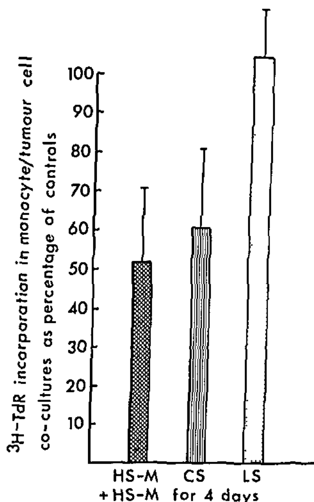


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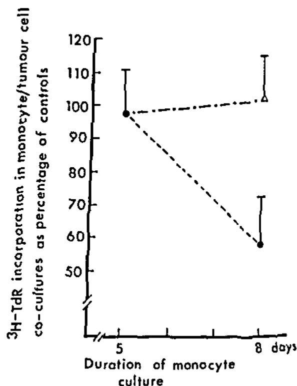


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Fig 7 Monocytes exposed to CS (A) and LS (B) from 72nd to 96th h and subsequently cultured in HS M for 4 days (× 400)

Acta path microbiol scand Sect C 87 333–340 1979

ADHESION AND LOCOMOTION OF HUMAN LEUKOCYTES *IN VITRO*, IMPORTANCE OF PROTEIN COATING, EFFECT OF LIDOCAIN, ETHANOL AND ENDOTOXIN

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Schreiner A & Hopen G Adhesion and locomotion of human leukocytes *in vitro* importance of protein coating effect of lidocain ethanol and endotoxin Acta path microbiol scand Sect C 87 333–340 1979

The adhesion of leukocytes to glass beads in protein free media was quantitatively high and not dependent on divalent cations Addition of plasma albumin or gelatin in increasing concentrations gradually reduced leukocyte adhesion which then became increasingly dependent on divalent cations Heat inactivation of plasma did not affect leukocyte adhesion Leukocyte migration in glass capillary tubes which was dependent on a heat labile plasma factor was promoted by each of the proteins listed and by siliconizing the tubes Leukocyte migration in millipore filters was enhanced when albumin was present in the cell starting compartment Lidocain reduced both leukocyte adhesion to protein-coated glass and leukocyte migration in capillary tubes and millipores filters Ethanol reduced leukocyte adhesion and leukocyte filter migration *E coli* endotoxin enhanced adhesion of leukocytes but inhibited their migration in tubes and filters The findings indicate the existence of a relationship between adhesion and migration of leukocytes

Key words Leukocyte adhesion leukocyte migration protein coating lidocain ethanol endotoxin

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Adhesion and migration of phagocytes are important features of the inflammatory response Since migrating phagocytic cells crawl on more or less solid surfaces variations in the adhesiveness of these cells may modify their locomotive capacity [illegible]

[illegible] of phagocytic cells (MacGregor *et al* 1974 *Rabinowitch & DeStefano* 1975 *Schneier et al* 1977) In the present work the influence on human leukocyte adhesion and migration of a number of factors was studied *in vitro* and the results compared The factors included were plasma albumin gelatin divalent cations lidocain ethanol and endotoxin

MATERIAL AND METHODS

Blood was donated by members of the laboratory staff and by medical students All blood specimens were heparinized to contain 10 i u per ml Casein Hammarsten and disodium EDTA were purchased from Merck Darmstadt West-Germany endotoxin (E coli 026 B6) and gelatin from Difco Laboratories Detroit, Mich U S A heparin from Apothekernes Laboratorium Oslo Norway human serum albumin (HSA) from Kabi Haslum Norway and lidocain from Astra Sodertalje Sweden. Glass beads were purchased from Dragon Werk Bayreuth West-Germany Micro-hematocrit capillary tubes from Modulohm Herslev Denmark modified Boyden chemotaxis chambers from Neuroprobe Bethesda Md U S A and millipore filters from Millipore Filter Corp Bedford Mass U S A Columns for the glass beads were made from commercial polyvinyl tubing Hanks balanced salt solution (HBSS) containing 12×10^{-4} M Ca^{++} and 8×10^{-4} M Mg^{++} was used as the main cell-suspending medium

Isolation of leukocytes Leukocytes were isolated by

been demonstrated by *in vitro* experiments with mouse cells (5)

The excellent technical assistance of *M Sorensen* and *A Remen* is gratefully acknowledged I am also indebted to Professor *J Lamvik* for discussion and help during the course of this study and in preparation of the manuscript This work was supported by grants form the *Norwegian Research Council for Science and the Humanities* the *Norwegian Cancer Society* and the *Norwegian Society for Fighting Cancer* The author is a research fellow of the *Norwegian Research Council for Science and the Humanities*

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Acta path microbiol scand Sect C 87 333–340 1979

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Adhesion and migration of phagocytes are important features of the inflammatory response Since migrating phagocytic cells crawl on more or less solid surfaces variations in the adhesiveness of these cells may modify their locomotive capacity Several cell researchers have reported that environmental factors such as proteins and divalent cations may influence strongly the adhesion of different cell types to artificial substratums (*Fenn* 1923 *Taylor* 1961 *Garvin* 1968 *Takeichi* 1971) Also drugs and bacterial factors may modify adhesion and locomotion of phagocytic cells (*MacGregor et al* 1974 *Rabinowitch & DeStefano* 1975 *Schneier et al* 1977) In the present work the influence on human leukocyte adhesion and migration of a number of factors was studied *in vitro* and the results compared The factors included were plasma, albumin gelatin divalent cations lidocain ethanol and endotoxin

MATERIAL AND METHODS

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was used as the main cell-suspending medium

Isolation of leukocytes Leukocytes were isolated by

dextran sedimentation and the contaminating erythrocytes were lysed with ammonium chloride, as described in detail elsewhere (*Schreiner* 1978)

Adhesion assay Adhesion was measured as percentage retention of leukocytes after passage through a glass bead column The polyvinyl column (length 80 mm, internal diameter 5 mm) contained approximately 11,500 glass beads with an average diameter of 0 5 mm, yielding a total glass surface of approximately 9×10^3 mm^2 A cell suspension containing 7×10^9 leukocytes per litre was perfused at a constant flow of 0 2 ml per min, and leukocyte counts were made in the first 0 5 ml eluate The columns were tilted slightly from the horizontal position in order to ease the evacuation of air bubbles The method is described in detail elsewhere (*Hopen & Schreiner* 1979)

Tube migration Migration of leukocytes on the inner surface of capillary glass tubes was studied, using a modification of the method described by *Ketchel & Favour* (1955) A suspension of leukocytes containing 7×10^9 cell/l was aspirated into hematocrit capillary tubes and the tubes were sealed by heat at one end and centrifuged at $2500 \times g$ for 3 min It was observed at the start of the study that before incubation, and in the absence of protein in the medium, leukocytes in relative abundance were attached to the inner surface of the capillary tube in its full length Therefore, in later experiments the leukocyte suspension was aspirated only to the mid-point of the tube, the tube was sealed at the opposite end, and the leukocytes were centrifuged down to that end In these tubes, when no protein or only a small concentration of protein was present, cells were seen to be stuck to the inner surface of the capillary tube in its upper half, and a haze of cells was observed above the solid leukocyte layer (Fig 1) With higher protein concentrations, no such haze was observed before incubation After incubation for 2 h at 37°C, the distance between the demarkation of the solid cell layer and the border of the cell haze was measured with an ocular micrometer and expressed in mm When measuring this distance, the ten most distant cells were disregarded Hereafter, the distance measured in this way is referred to as the »cell front size« In some experiments the cell front present *before* incubation was also measured

Millipore filter migration The rate of granulocyte filter migration was determined, using modified Boyden chemotaxis chambers and three micrometer pore size millipore filters The upper compartments contained a suspension of 2.5×10^9 leukocytes/l in HBSS with 2% HSA, either alone or combined with a test substance The lower compartments contained approximately 4 mg per ml casein in HBSS In the endotoxin experiments, equal concentrations of endotoxin were present in both compartments The rate of migration was determined by counting cells that had reached a section of the filter 75 micrometers from the cell-starting side after incubation for 2 hrs The method has been described in detail previously (*Schreiner* 1978)

Statistics The Wilcoxon rank sign test was used for the statistical calculations

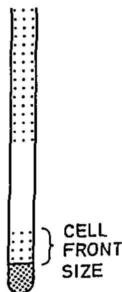


Fig 1 Leukocytes attached to the inner surface of a capillary tube after centrifugation when protein free leukocyte suspension had been aspirated half way into the tube

RESULTS

Influence of plasma on adhesion and migration Experiments were made with varying concentrations of plasma The leukocyte adhesion, when measured as retention in glass bead columns, became increasingly reduced as the plasma concentration was increased Addition of 10 mM EDTA to chelate divalent cations had little effect on the retention of leukocytes in the absence of plasma However, the inhibitory effect of EDTA on retention increased with increasing plasma concentration and was complete with undiluted plasma (Fig 2a) In the tube migration experiments, a front of cells, which decreased with increasing plasma concentration and reached zero at 10% plasma, was observed *before* incubation After incubation, the distance measured from the solid cell layer to the ten leading cells in tubes with 10% and 100% plasma had increased significantly, indicating a true migration, whereas the size of the cell front in tubes with 0 and 1% plasma was unchanged (Fig 2 b) When the experiments were repeated with addition of EDTA, there was no significant difference in the cell front size before or after incubation at any plasma concentration studied (Fig 2 c)

The retention in glass bead columns of leukocytes suspended in plasma which had been heated for 30 min at 56°C was not significantly different from the retention of leukocytes suspended in untreated plasma (n = 7, p > 0 1, not shown) In capillary

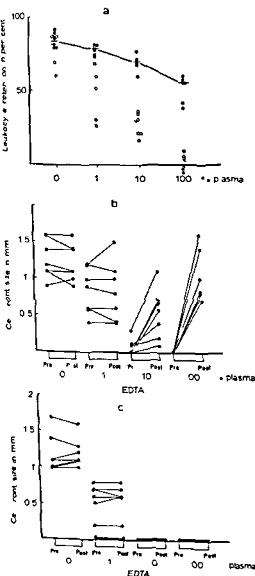


Fig 2 Influence of plasma.
a) on retention (adhesion) of leukocytes in glass bead columns in the absence (●——●) and presence (O——O) of EDTA Individual observations and median
b) on the cell front size in the absence of EDTA
c) on the cell front size in the presence of EDTA
Pre before post after incubation

tube experiments with leukocytes in heat inactivated plasma, no migration could be observed (n = 7 *not shown*)

Influence on adhes on and migration of album n and gelat n Glass bead columns and capillary tube experiments were made using leukocytes suspended in HBSS to which different concentrations of HSA or gelatin had been added In glass bead columns the retention of leukocytes decreased as the concen tration of each protein was increased Addition of EDTA led to a further reduction of the retention in each case (Fig 3 a and b) In the capillary tube experiments the cell front present before incubation was increasingly reduced as the concentration of each additive was increased The size of the cell front came close to zero at 10% HSA and at 0 5% gelatin After incubation no change in the cell fronts had taken place except in some experiments using 0 5% or 1% gelatin in which a small increment could be observed (Fig 3 c and d)

Effect on adhesion and migration of the combina tion of plasma and gelatin In glass bead columns the retention rates were significantly ($p < 0.05$) reduced when a combination of 0 5% gelatin and 5% plasma was added to the medium as compared to retention rates with 5% plasma alone (Fig 4 a)

In capillary tubes with leukocytes suspended in 5% plasma leukocyte migration was enhanced significantly ($p < 0.01$) when 0 5% gelatin was added to the medium (Fig 4 b)

Influence on adhesion and migration of siliconi zing glass material Siliconizing of the glass beads in column experiments reduced the leukocyte retention in a protein free medium ($p < 0.01$) The leukocyte retention was significantly more inhibited ($p < 0.01$) in the presence than in the absence of EDTA (Fig 5)

Capillary tube migration of leukocytes suspended in 5% plasma was significantly greater ($p < 0.01$) in siliconized than in non siliconized tubes Using cells suspended in 100% plasma, siliconizing did not change ($p > 0.1$) the rates of migration (Fig 6) In experiments using siliconized tubes and leukocy tes suspended in HBSS to which 0 1% HSA had been added a marked cell front was observed before incubation (n – 7 median cell front size 0 6 mm range 0 5–0 7 mm *not shown*) After incubation no change n the cell f [illegible]

filt [illegible]
cas [illegible]

medium in the upper compartment significantly enhanced migration as compared to experiments where HBSS alone was present above the filter ($p < 0.05$) When the filters were soaked with 2% HSA and gently rinsed in HBSS migration in these filters did not differ ($p > 0.1$) from migration in filters w h HBSS [illegible]

a

Leukocyte retention in per cent

100

50

0 01 1 10 % HSA

b

100

50

0 01 05 1 % gelatin

c

Cell front size in mm

2

1 5

1

0 5

Pre Post Pre Post Pre Post Pre Post

0 01 1 10 % HSA

d

2

1 5

1

0 5

Pre Post Pre Post Pre Post Pre Post

0 01 05 1 % gelatin

Fig 3 Influence of human serum albumin and gelatin on
a) and b) Leukocyte retention (adhesion) in glass bead columns in the absence (● —— ●) and presence (O - - - - O) of EDTA Individual observations and median
c) and d) cell front size in capillary tubes
Pre before post after incubation

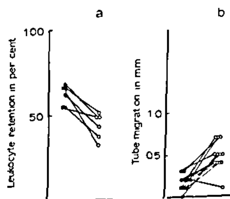


Fig 4 Effect of the *combination* of 5% plasma and 0 5% gelatin (O) as compared to 5% plasma alone (●) on
a) leukocyte *retention* (adhesion) in glass bead columns
b) leukocyte migration in capillary tubes

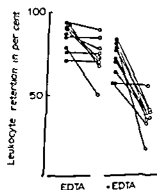


Fig 5 Leukocyte retention (adhesion) from protein free leukocyte suspensions in columns with siliconized glass beads (O) as compared to unsiliconized controls (●) in the absence and presence of I

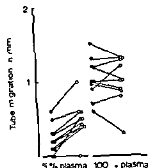


Fig 6 Migration of leukocytes suspended in 5% or 100% plasma in siliconized capillary tubes (O) as compared to unsiliconized controls (●)

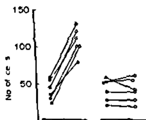


Fig 7 Migration of leukocytes in Boyden millipore filters when Hanks solution alone (●) or 2% human serum albumin in Hanks (△) was present above the filter and when the filters had been soaked in 2% human serum album n and gently rinsed (O) (Hanks alone above the filter)

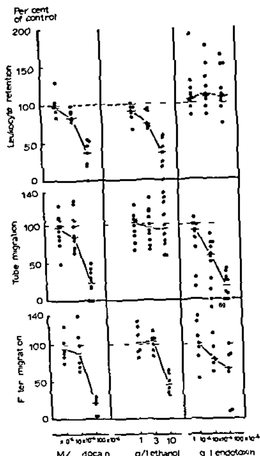


Fig 8 Effect of different concentrations of lidocain ethanol and endotoxin on leukocyte retention (adhesion) in glass bead columns leukocyte migration in capillary tubes and leukocyte migration in Boyden millipore filters Individual observations in % of paired controls and median For glass bead column and capillary tube experiments the leukocytes were suspended in 90% plasma

bead columns Leukocyte migration in tubes and in millipore filters was not influenced by 10×10^{-4} M lidocain ($p > 0.1$) but was significantly reduced by 100×10^{-4} ($p < 0.02$)

Ethanol in increasing concentrations produced an increasingly reduced retention in glass bead columns Leukocyte tube migration was not influenced by 10 g/l ethanol ($p > 0.1$) but leukocyte filter migration was significantly reduced at this concentration ($p < 0.05$)

Endotoxin in concentrations 10×10^{-4} and 100×10^{-4} g/l *significantly* increased leukocyte retention in glass bead columns ($p < 0.05$) Leukocyte migration in tubes and filters was increasingly reduced as the endotoxin concentrations were increased However in filter experiments this reduction was significant ($p < 0.05$) only at 100×10^{-4} g/l endotoxin (Fig 8)

DISCUSSION

Already in 1923 *Fenn* demonstrated that leukocyte adherence to solid surfaces was influenced by environmental factors such as the presence of serum ion concentration and temperature. Based on extensive studies under non flow conditions of adhesion of human conjunctiva cells to various solid surfaces *Taylor* (1961) suggested the existence of two different types of contact After contact with naked glass cells attached and spread rapidly Because this kind of adhesion was strong resisted attempts at reversion by physical forces and was not dependent on divalent cations or cell viability it was considered to be of a passive physico-chemical

nature A fundamentally different type of adhesion occurred in the presence of serum or serum fractions, or to surfaces which had been coated with protein or other macromolecules Under these conditions, cells attached less strongly and spread more slowly This type of adhesion, which was reversible by mechanical forces and dependent on divalent cations and cell viability, was considered to be an active, biological process performed by the living cell The results of successive studies of cell adhesion strongly support *Taylor's* hypothesis *Takeichi* (1971) found under non-flow conditions a firm attachment of chick fibroplasts to clean plastic which was not dependent on divalent cations In contrast, the weaker adhesion to coated plastic was *dependent on divalent cations In Garvin's* experiments (1968) made under flow conditions, rat leukocytes adhered completely to glass and were not dependent on divalent cations Adhesion of these leukocytes was reduced markedly and became dependent on divalent cations when either protein had been added to the cell suspending medium, or when the glass surface had been pre-coated with protein These findings were reproduced in our laboratory using human leukocytes (*Hopen* unpublished work) *Kvarstein* (1969) demonstrated under flow conditions that inhibitors of glycolytic and oxidative cell metabolism depressed human leukocyte adhesion to glass in the presence of blood proteins

When studying cell adhesiveness, it is obviously important to know what type of contact is active Under physiological conditions, blood or blood proteins will always be present in the environment *Such conditions can be reproduced in vitro* by working with whole blood, or by adding blood proteins to the cell suspending medium, which will coat the substratum in seconds (*Bruck* 1977) Nevertheless, in several works on leukocyte function the importance of *protein coating has attracted* little attention

The results of our experiments with human leukocytes show that the adhesion of these cells to glass beads can be modified significantly by the presence of proteins and *that the adhesion may be a* combination of the two types of contact suggested above With leukocytes in protein-free HBSS, adhesion is almost total As plasma is added in increasing concentrations, the retention, *i e* adhesion, subsides When in these *experiments divalent* cations are chelated by adding EDTA the adhesion decreases more rapidly and is *totally inhibited* in undiluted plasma Apparently, the adhesion of leukocytes in the plasma free medium which is less dependent on divalent cations, is *dominated* by the passive, physico-chemical type of contact The adhesion of cells in pure plasma was totally dependent on divalent cations, and is therefore likely to be of the active, biological type Both types of *contact* seemed to act simultaneously in experiments with 1% and 10% plasma, since in these cases the adhesion could be partly inhibited by adding EDTA Conceivably, the difference between measurements with and without EDTA might represent the rate of active adhesion Labile plasma factors did not appear to be critically involved with active adhesion, since *heating the plasma did not change* the retention rates Reduction of wettability by siliconizing the columns reduced the rate of passive adhesion but far from abolished it When experiments were repeated with HSA or gelatin, the leukocyte adhesion was reduced increasingly as the concentration of each protein was increased The difference in retention rates in experiments with and without EDTA for each protein showed that in each case some degree of active adhesion had taken place, though this was less than when plasma was used Consequently, provided that divalent cations are *present, active adhesion takes place also in the* absence of plasma

The decreasing rate of leukocyte adhesion to glass at increasing protein concentration is illustrated in our capillary tube experiments In experiments with no protein, or with low protein *concentrations* present, a front of cells attached to the inner glass wall above the solidly packed cells was observed before incubation Apparently this front had been formed by cells that had been slowed down by crowding during centrifugation and thereby allowed to adhere to the glass wall The size of the cell front – which was not dependent on the presence of divalent cations – decreased when the protein concentration in the medium was *raised* and reached zero for different concentrations of each protein (Fig 2 b c, 3 c, d) The findings from both *the glass bead* and capillary tube experiments may be due to protein coating of the substratum to which the leukocytes adhere As the protein concentration is raised the protein interface between cell and glass increases in thickness (*Hayry* 1966) and the rate of *passive adhesion decreases* accordingly Hence in glass bead columns the active adhesion becomes measurable In capillary tubes protein coating increasingly prevents cells from adhering to the glass wall

After incubation of the capillary tubes the cell front had increased significantly in experiments with 10% or more plasma, thus indicating migration The migration was dependent on divalent cations With heat-inactivated plasma, HSA or gelatin, only minimal or no migration at all, had taken place This dependence of capillary tube

migration on a heat labile plasma factor was also found by *Ketchel & Favour* (1955)

Some degree of adhesion is a prerequisite for the automobility of a body on a solid surface Therefore the relationship between leukocyte adhesiveness and leukocyte locomotion seems to be of interest An increased adhesive force may exceed the propelling force and thus immobilize the leukocyte Moreover a reduced adhesiveness may become insufficient to yield reaction to the propelling force and thereby prevent propagation of the cell In addition to being dependent on a plasma factor the migration in our capillary tubes was promoted by the presence of protein. Migration of leukocytes in 5% plama increased significantly when 0 5% gelatin had been added to the medium This can be explained either by a reduction in glass adhesion by the additional protein and consequent liberation of propelling forces or by a reduction in cell to-cell adhesion caused by the proteins present Our finding that leukocytes suspended in 5% plasma migrated better in siliconized than in non-siliconized tubes indicates tha [illegible] rol [illegible] the [illegible] tion in 100% plasma corresponds well to results reported by *Weiss & Blumenson* (1967) in which sil conizing had no effect on cell adhesion in the presence of undiluted plasma Both findings indicate that sil conizing is no obstacle to the inhibition of glass adhes [illegible]

[illegible] claimed that chamber (millipore filter) migration and tube migration involve separable mechanisms of cell function His conclusion was based on capillary tube experiments with siliconized tubes and 0 1% HSA in the cell suspending medium As demonstrated in previous (*Ketchel & Favour* 1955) and present experiments a plasma factor is required for leukocyte migration in capillary tubes In addition, the presence of only 0 1% HSA is insufficient to keep the adhesion to glass from acting strongly on the leukocytes even in sil conized tubes In the light of our findings an explanation might be that the front of cells observed by *M ller* consisted of cells that had adhered to the glass wall before incubation and were later mistaken for cells that had m grated Under such circumstances EDTA and the metabol c inhibitors used by *M ller* would have no influence

Wilkinson & Allan (1978) reported that bovine and human serum albumin is chemokinetic for leukocytes and suggested that albumin is necessary for the leukocyte response to chemotactic stimulation by low molecular weight attractants In our Boyden experiments the granulocytes migrated significantly better against the high molecular weight casein when albumin was present above the filter than when it was absent Possibly this difference may be explained as an increase in leukokinesis even though it takes place in a negative gradient of albumin An attempt to explain the increased migration as a result of the filter structure being coated with albumin was not successful Migration in filters which had been soaked with HSA an rinsed in HBSS was not different from migration in filters treated with HBSS alone A supplement to *Wilkinson s* report on the effects of albumin migt be that albumin in the cell suspending medium helps to prevent cell to-cell adhesion and thereby facilitates the migration of single cells into the filter In our Boyden experiments without albumin or other proteins in the medium cell clumping is a constant obstacle. Migration in these experiments is weak and the results are less reproduceable (*Schreiner* unpublished work)

Lidocain is reported to inhibit the adhesiveness of macrophages (*Rabinowitch & DeStefano* 1975) In our experiments lidocain reduced leukocyte adhesion to protein-coated glass beads and inhibited leukocyte migration in tubes and filters However a reduction was measured at lidocain concentrations which occur only after local application of the anaesthetic

Ethanol reduced leukocyte adhesiveness in concentrations which are seen after ethanol intake while tube migration was uninfluenced by the concentrations used and filter migration was reduced only by concentrations which are extremely toxic to humans Our results with ethanol are in accordance with the studies made by *MacGregor et al* (1974) and *Spagnuolo & MacGregor* (1975) who used different adhesion and chemotaxis assays *E coli* endotoxin which is reported to increase leukocyte adhesiveness (*Schneier et al* 1977) did so also in our glass bead experiments However as with ethanol and lidocain a reduction in tube and filter migration also resulted when using endotoxin.

The results of our studies lead to the following conclusions The nature of leukocyte adhesion to glass is dependent on whether or not protein is present The type of contact to glass occurring in the absence of protein is a physico-chemical process which is unlikely to have a physiological significance Under *in vitro* conditions the cell to-glass adhesion can be reduced gradually by increasing the protein concentration Leukocyte migration in glass capillary tubes can also be reduced or totally inhibited by different degrees of the physico-chemical type of contact and is maximal only when

this type of adhesion is prevented by sufficient protein coating Active adhesion of leukocytes is dependent on divalent cations but is not affected by the destruction of heat labile plasma factors Some degree of active adhesion may also be observed in the absence of plasma

The finding that by increasing the protein coating a reduction in adhesion of leukocytes could be observed parallel to an increase in leukocyte tube migration indicates that a relationship may exist between the two leukocyte functions If an optimum of cell adhesiveness for the promotion of leukocyte migration does exist the finding that both increment and reduction of leukocyte adhesiveness induced by the agents tested were combined with impaired migration in at least one system gives rise to the assumption that this optimum might be carried by uninfluenced leukocytes

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Acta path microbiol scand Sect. C 87 341-345 1979

THE INFLUENCE OF SPECIFIC ANTIBODIES AND CELLULAR IMMUNITY IN THE INDUCTION OF TOLERANCE TO CHEMOTACTIC ACTIVITY CAUSED BY *BACTEROIDES FRAGILIS* LIPOPOLYSACCHARIDE

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Sveen K & Hofstad T The influence of specific antibodies and cellular immunity in the induction of tolerance to chemotactic activity caused by *Bacteroides fragilis* lipopolysaccharide Acta path microbiol scand Sect. C 87 341-345 1979

Exudate aspirated from wound chambers implanted subcutaneously on rabbits at different time intervals after local lipopolysaccharide (LPS) injection showed a peak of chemotactic mediator concentration *in vitro* about two hours before the peak of polymorphonuclear lekocytes accumulated *in vivo* was demonstrated Injection of LPS locally into the wound chambers three days after the first injection of LPS showed a reduced number of PMNs accumulated in the exudate Antibodies to the LPS preparation were demonstrated in the exudate and serum by indirect haemagglutination of sheep erythrocytes before the second LPS injection This antibody titre increased up to two weeks after the first LPS injection and was slightly higher in the serum than in the exudate Also a migration inhibition factor (MIF) activity was demonstrated in the exudates formed This MIF activity of the exudates increased after the second LPS injection The increased titre of specific antibody may indicate an accelerated clearance of LPS and the MIF activity may indicate a reduced response of PMNs to chemotactic mediators However the involvement of other biological mechanisms contributing to the decreased response cannot be excluded

Key words *B fragilis* lipopolysaccharide antibody cellular immunity chemotaxis tolerance

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Repeated inoculations of bacterial lipopolysaccharides (LPS) into man and animals induce tolerance viz refractoriness or increased resistance to the endotoxic effects of the LPS Tolerance is relative and fever and other host responses can be elicited by increasing the dose of LPS (5) Cross tolerance to endotoxic LPS of heterologous bacterial species is present but this tolerance is significantly lower than that directed against the homologous LPS preparation (6) Two phases determined by separate mechanisms are involved in tolerance to endotoxin the early cellular refractory state being transient and a later phase of refractoriness which may be mediated by specific antibodies (6 7)

Mediators for leukocyte chemotaxis are generated in the fluid of wound chambers implanted subcutaneously on rabbits after injection of LPS (15, 17, 20) Upon repeated injections of LPS a decrease in chemotaxis takes place indicating some form of tolerance The present study is concerned with this phenomenon and with the possible mechanisms underlying it It is shown that the reduced number of leukocytes accumulated in the wound chamber fluid after repeated injections of LPS is not solely due to a reduced elaboration of leukotactic mediators The LPS used in the experiments was isolated from a strain of *Bacteroides fragilis* The toxic

MATERIALS AND METHODS

Lipopolysaccharide

LPS was isolated from *Bacteroides fragilis* strain Lille E 323 by extraction with phenol-water (23), and purified by ultracentrifugation (13) Stock suspension of LPS-E 323 containing 5 mg per ml was made in sterile isotonic saline This was treated ultrasonically (MSE/Mullard, 60W, 20 kc/s) at 0°C for 30 s

Rabbit Polymorphonuclear Leukocytes

Polymorphonuclear leukocytes (PMNs) were obtained from the peritoneal cavity of New Zealand White rabbits 8–10 h after intraperitoneal injection of 200 ml of a solution of 0 1 per cent glycogen (E Merck AG, Darmstadt, W-Germany) in sterile isotonic saline Garamycin® (Schering Corporation, Bloomfield, N J, USA) and fungizone® (Squibb, Flow Laboratories, Irvine, Scotland) were added in amounts of 50 and 2 5 [illegible]

in the same concentrations as in the glycogen solution *Bovine serum albumin* (Armour Pharmaceutical Company Ltd , Eastbourne, England) (2 per cent w/v) were then added and the medium adjusted to a concentration of 10^7 cells per ml

Measurement of Leukocyte Migration in vitro

The migration of PMNs was determined by the Boyden micropore technique (2), using a modified Boyden chamber (12) (Neuroprobe, Bethesda, Md , USA) The upper compartment of the chamber received approximately 3×10^6 cells These were first centrifuged down on a Millipore membrane (Neuroprobe) with a *pore size of 3 μ (14) The different test media containing* either LPS or exudate, were placed in the lower compartment The chambers were incubated at 37°C for 3 h in humid air The filters were fixed, stained and mounted as previously described (14) Five fields on the chemotactic side of the filters were selected for counting at a magnification of × 320 with the aid of a microgrid (*Leitz Wetzlar 10 × 10 mm*)

Measurement of Leucocyte Migration in vivo

Five Teflon® chambers were implanted subcutaneously on each lateral side of the abdomen of six-month old New Zealand White rabbits (20) Six days after the implantation of the chambers, the wound fluid formed was aspirated (Exudate I) and suspensions of 100 μg of LPS E 323 in 0 4 ml of sterile isotonic saline *were injected into each chamber* At zero ½ h 1 h and every hour thereafter until the eight hour after the application of LPS the exudate (*Exudate II*) was aspirated from one chamber and the accumulation of leukocytes per μl of exudate was determined as described previously (20) Three days after the first injection of LPS the exudates in all chambers (Exudate III) were *thoroughly* aspirated and the number of PMNs per μl of exudate calculated Thereafter, 100 μg of LPS in saline was reinjected *into each chamber*, and the number of PMNs per μl of exudate (Exudate IV) was determined at the same time intervals as for Exudate II Three days after the second stimulation (on day 12 after the implantation of the chambers), the wound chamber fluid formed was again aspirated (Exudate V), and the number of cells accumulated per μl of exudate determined The last sampling of wound chamber *fluid was performed* four weeks after the implantation (Exudate VI) Each *time the collected exudates* were immediately chilled to 0°C After centrifugation of the exudates at 28 000 × *g* for 30 min at 4°C (Servall RC-2, Sorvall Inc , Norwalk Conn , USA), the supernatants were pipetted off, then inactivated at 56°C for 30 min and merthiolate (0 1 per cent *w/v) was added before they were stored at –25°C* until used

Antiserum

Antiserum to LPS-E 323 was produced in rabbits by *injection of whole organisms (9) The antiserum was* inactivated at 56°C for 30 min in a water bath and merthiolate (0 1 per cent w/v) was added before it was stored at –25°C until used

Indirect Haemagglutination

The antiserum to *B fragilis* E 323 was absorbed with sheep erythrocytes before use These were sensitized with LPS-E 323 which had been treated with 0 25 M NaOH for 1 h at 56°C and neutralized with 0 25 M HCl Erythrocytes in a 1 per cent suspension in phosphate buffered saline, pH 7 2 (PBS) were sensitized (1 h at 37°C) with *an equal volume of LPS (12 5 μg per ml)* Indirect haemagglutination (HA) was carried out in U-well plastic trays (Microtitre equipment, *Flow Laboratories*, Irvine, Scotland) as described previously (10) The trays were kept at room temperature for 1 h, and stored at 4°C overnight The patterns were then read

Migration Inhibition Test

Guinea pig peritoneal exudate cells were used Five [illegible]

rium for Specialpræparater Oslo) and the cells isolated *(14) Differential count of the cells showed more than 70* per cent macrophages, the other cells being primarily lymphocytes The cells were transferred to *heparinized* microhaematocrit capillary tubes and three of these were put in petri dishes (4 5 cm diameter) and held in place by a small amount of silicone Each exudate sample (0 5 ml) (Exudate I III V or VI) was mixed with 3 ml of Eagle s medium (GIBCO Grand Island N Y USA) The Eagle s medium contained 100 I U /ml penicillin (Apo*thekernes Lab Oslo) 100 μg/ml of streptomycin* (Lederle Lab Div Pearl River N Y USA) and 5 per cent *heat inactivated foetal calf serum Each exudate was then* placed in 3 petri dishes and tested with Exudate I serving as the reference (negative *control*) After incubation at 37°C in humid air containing 5 per cent CO_2 for 6 h the petri dishes were placed under an inversion microscope and photographed by a Polaroid camera using a four-fould magnification objective The area of migration was

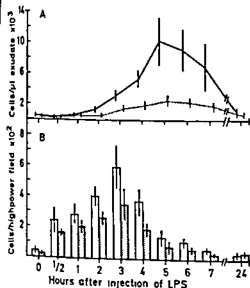


Fig 1 Upper panel (A): Kinetics of leukocyte accumulation in wound chambers after local injection of 100 µg of LPS-E 323 in 0 4 ml saline Heavy line after the first injection of LPS Broken line second injection (three days after the first injection) of LPS Each point represents the mean . .

. p < 0 06) (two-sample t test) Lower panel (B) leukocyte chemotaxis from the same chamber exudate as shown in the upper panel tested in a modified Boyden chamber device Each column represents the mean number of cells on the chemotactic side of the filter ± standard deviation (vertical bar) from three filters Open columns exudate after first injection of LPS Cross hatched columns exudate after the second injection of LPS The chemotactic act . . . injection . . . second . . . p < 0

calculated by planimetri and the percentage og migration inhibition calculated (19) Migration inhibition of more than 25 per cent was regarded as significantly (19)

Double D ff . . .

. . . wells with diameters of 3 mm at a distance fr . . . for . . . prec . . .

Calculation of Haemagglutination Titre

The mean haemagglutination titres were determined by numbering the titres 10 = 1 20 = 2 40 = 3 etc The titre halfway between the dilution giving haemagglutination and the next dilution step was chosen as the determining titre (13)

RESULTS

The kinetics of leukocyte accumulation in the wound chambers in response to LPS-E 323 is shown in Fig 1A After 5 h, an optimal number of cells had accumulated, both after the first and after the second local application of endotoxin After the second injection, however, the number of cells at the summit of the bell-shaped time-response curve observed after 5 h was significantly lower than that found after the first injection of LPS ($p < 0.001$) If a third local injection of LPS was given, the time-response curve was essentially the same as that seen after the second injection, but even fewer cells accumulated in the exudate

Exudates aspirated at different times after injection into the chamber of LPS were tested in Boyden chambers for chemotactic activity Maximal accumulation of cells on the chemotactic side of the filter was obtained with exudates aspirated 3 h after LPS injection (Fig 1B)

Serum and exudate (Exudate I) obtained before the injection of LPS into the chambers did not contain antibodies that agglutinated sheep erythrocytes sensitized with NaOH treated LPS-E 323 A peak of antibody activity was found in the exudates aspirated 6 days after the first injection of LPS (Exudate V) as well as in samples of serum taken at the same time (Fig 2) Three weeks after the first injection of LPS, the antibody titre of the exudate (Exudate VI) had decreased almost four-fold,

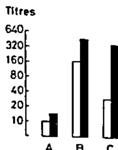


Fig 2 Titres in the indirect haemagglutination test of exudates (open columns) sampled at different time intervals after injection of 100 µg of LPS E 323 in 0 4 ml of saline into each wound chamber Closed columns illustrate sera sampled at the same time intervals A) three days B) one week and C) three weeks after the first injection of LPS Each column represents the mean titre from four rabbits

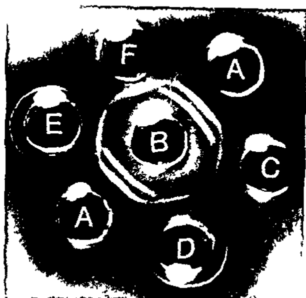


Fig 3 Double diffusion in agar gel Wells A contain antiserum against LPS-E 323 (well B) Exudate V in well C and corresponding serum sample in well D Wells E and F contain Exudate VI and corresponding serum sample, respectively

whereas the corresponding serum sample revealed nearly the same activity as that observed 6 days after the initial injection of LPS

One line appeared when exudates V and VI and corresponding serum samples were tested by precipitation in agar against LPS (Fig 3) No precipitation line was seen when testing Exudate III or the corresponding serum sample

A migration inhibition activity was demonstrated in all exudates after the first injection of LPS into the tissue chambers (Table 1)

DISCUSSION

The finding that chemotactic mediator activity is highest in the exudates in response to injection of LPS 2 h before maximal accumulation of PMNs is in agreement with earlier observations (16) In fact, chemotactic mediator activity was demonstrated *in vitro* before demonstration of any cell accumulation *in vivo* These results are also in agreement with those reported earlier (18, 20) and this experiment gives valuable information about the rate at which the PMNs are mobilized at different time intervals after an inflammatory state is initiated Thus, a bell-shaped curve of time versus cellular response seems to be typical for the migration of PMNs into an inflammatory focus

The chemotactic mediator activity of the exudates after the second injection of LPS, and the decrease in number of cells accumulated, show that tolerance to the chemotactic effect of *B fragilis* LPS can be induced Several mechanisms may be operating The production and/or activity of the chemotactic mediator, which is possibly the complement factor C5a (17, 18), may be inhibited or inactivated

The role played by specific antibodies is difficult to assess Conventional LPS is bound initially to leukocytes and platelets (3), and subsequently taken up by macrophages (4) This is a slow-acting process taking hours During this period, LPS taken up by macrophages is transferred to lymphocytes (1) The antibody activity to *B fragilis* LPS demonstrated in both the exudate and the serum 3 days after the first injection of LPS and which showed a peak after 6 days, indicates that an antibody-mediated increase in clearance of LPS from the chambers may be involved in the mechanisms leading to the decrease in chemotactic activity This would imply an accelerated phagocytosis of LPS by opsonizing antibodies However, the less significant difference in chemotactic mediator activity of the exudates aspirated from the chambers after the first and second injection of LPS demonstrated *in vitro* (cf Fig 1B), indicates that an *in vivo* induction of mediator formation takes place after the second injection which is nearly of the same strength as that following the first injection of LPS This again suggests that specific antibodies do not contribute to tolerance simply by accelerating the clearance of LPS by phagocytes On the other hand, immune complexes formed by LPS and specific antibodies may contribute to leukocyte chemotaxis by generation of mediators through activation of the classical complement pathway

Pilot experiments carried out in our laboratory have shown that injection into the chambers of *Veillonella alcalescens* LPS 3 days after the injection of LPS from *B fragilis* E 323 evokes a cross-tolerance LPS of *V alcalescens* is a typical LPS (8), with which *B fragilis* LPS has no lipid A or polysaccharide core structure in common Thus, the two LPS have not been found to cross-react through their O antigens This pilot finding might indicate that the production of antibodies is not the only humoral factor in the induction of tolerance to the chemotactic activity induced by *B fragilis* LPS

LPS directly stimulates B-lymphocytes to produce antibodies It is also well known that T-lymphocytes stimulated with antigen produce lymphokines which are the specific humoral mediators in the cellular immune response Under special conditions, B-lymphocytes may produce lumphokines such as the migration inhibition factor (MIF) (11, 24), acting on both macrophages and polymorphonuclear leukocytes and the chemotactic

factor (CF) (22) The demonstration of a low number of cells accumulated in the chamber exudates after the second injection of LPS in spite of a relatively high chemotactic mediator activity, as estimated by the *in vitro* experiments may also suggest that the PMNs were inhibited from migrating into the exudate The significantly increased migration inhibition, as expressed by these

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ISOELECTRIC FOCUSING OF MACROPHAGE CULTURE MEDIA AND THE EFFECT OF THE FRACTIONS ON THE SYNTHESIS OF DNA AND COLLAGEN BY FIBROBLASTS

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Jalkanen M Peltonen J & Kulonen E Isoelectric focusing of macrophage culture media and the effect of the fractions on the synthesis of DNA and collagen by fibroblasts Acta path microbiol scand Sect C 87 347–352 1979

Macromolecules from rat peritoneal macrophage culture media were separated into 30 fractions by flat bed isoelectric focusing (IEF) The fractions were tested for their influence on thymidine and proline incorporation into cultured rat granuloma fibroblasts Three fractions stable after freezing and lyophilization were of interest one inhibiting thymidine incorporation (focusing at pH 7 3–7 6) another stimulating thymidine incorporation (focusing at pH 4 4–5 3) and the third stimulating proline incorporation into cells and medium collagen (focusing at pH 10 2–10 7) The last also exhibited a ribonuclease (RNase) activity with a pH-optimum of 7 0–7 5

Key words Collagen synthesis DNA synthesis fibroblasts macrophages protein synthesis

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Macrophages secrete a variety of macromolecules not yet characterized chemically (5 12 18) Macrophages have been shown to facilitate the fibroblast proliferation in wound repair presumably by factors found in culture media of macrophages (12 13 18) Many hydrolytic enzymes are also known to be secreted by macrophages into culture media (7)

A common macrophage-derived promoter substance has been suggested for the growth of clones of both antigen triggered B lymphocytes and carcinogen triggered epithelial cells (20) On the other hand macrophages have also been shown to produce a factor which inhibits the cell proliferation in culture (4) and it has been demonstrated that activated macrophages can kill tumour cells through an intrinsic cytotoxic effector system (6)

Besides having an effect on cell proliferation macrophages also influence the protein synthesis in fibroblasts for instance in experimental silicosis (9) In earlier reports from our laboratory, a macrophage-derived factor has been shown to increase collagen synthesis *in vitro* in granuloma slices (1) and in cell free system (2) This factor is presumed to involve a ribonuclease (RNase) activity which possibly controls the stability of polysomes of *fibroblasts* (2)

The diversity of the functions connected with macrophages suggest that they secrete a number of factors [illegible] effects [illegible] cells an [illegible] culture conditions on the cell cycle of fibroblasts (16) A fractionation of the different macrophage-derived macromolecules thus seemed to be a fruitful even necessary, approach We have carried out flat bed isoelectric focusing (IEF) on macrophage medium molecules greater than 1800 daltons in the pH range 3 0–10 7, and have found three separate fractions which have biological effects on cultured rat granulation tissue fibroblasts

MATERIALS AND METHODS

Macrophage Cultures

Macrophages were prepared by washing the peritoneal cavity of adult Wistar rats with 20 ml of 0 9% (w/v) NaCl containing 10 units of heparin/ml (oy Star Ab, Tampere, Finland) The cells were washed with and suspended in Dulbecco's modification of Eagle's minimum essential medium (DMEM) (Flow Laboratories, Irwine, U K), buffered with 20 mM HEPES (Sigma Co , St Louis, Mo , U S A) and 22mM bicarbonate, and containing 100 U G-penicillin/ml and 50 µg streptomycin sulphate/ml After an incubation for 2 h in Nunclon 81-cm² cell culture flasks (N1470, A/S Nunc, Roskilde, Denmark), the macrophages (30–50 × 10^6 cells/10 ml) were washed with the medium to remove non-adherent cells, and fresh medium was added to the macrophages The macrophages were then incubated for 48 hours at 37 °C in air containing 5% CO_2, and the culture medium was collected and filtered through a cellulose ester filtration membrane (0 22 µm pore size Millipore Corp , Bedford, Mass , U S A) and replaced with fresh medium for another 48 hours

Fibroblast Cultures

Fibroblasts were isolated from 1–2 week-old experimental granulomas induced in adult rats of Wistar strain with a viscose cellulose sponge (21) by digestion of the sliced granulomas with collagenase and trypsin (10) Cells were cultured in DMEM supplemented with 10% foetal calf serum (FCS) (Flow Laboratories) under the conditions described above for macrophages Early subcultures from the 2nd to the 7th passages were used to assay the effects of the factors secreted by macrophages and fractionated by preparative isoelectric focusing

Isoelectric Focusing

Small molecules of macrophage culture media were removed by gel filtration in a BIO GEL 2 P column (Bio-Rad Laboratories Bromley U K , exclusion limit 1800 daltons eluant 5 mM phosphate buffer pH 7 3) The combined volumes of 50 or 250 ml of the 0–48 and 48–96 h media were then desalted and lyophilized After resolubilization and redesalting, Ultrodex gel (LKB Stockholm Sweden, final concentration 50 mg/ml) and Ampholine® (4 ml pH-range of 3 5–10 and an additional 1 ml pH range of 6–8 per 100 ml of the final gel slurry) were added to the macromolecule solution The focusing was performed in a LKB 2117 Multiphor apparatus as advised by the manufacturer The separation length of the gel was 23 cm width 11 cm and thickness approx 0 4 cm Focusing time was 17 hours with a voltage from 220 to 1000 V and current varying 20 to 8 mA The gel was cooled continuously with running water (+ 6 ° C) The focused gel was cut with a grid into 30 equal transverse fractions which were collected after measurement of the pH with a surface glass electrode The focused macromolecules were eluted from each of the gel portions with 10 ml of distilled water, lyophilized dissolved into DMEM (pH adjusted to 7 3), filtered through Millipore membrane (0 22 µm) and stored at + 4 ° C until used in biological assays Assuming that no significant losses occurred during the preparation 2 5 µl of a fraction in 200 µl of DMEM in a test well equalled the original concentration of macromolecules in the macrophage culture medium We used 0–50 µl of a fraction per 200 µl DMEM in the various experiments

Assays

Incorporation of ³H-thymidine and ³H-proline into fibroblasts For preparation of the test cultures approximately 7500 trypsinized fibroblasts in 200 µl DMEM containing 10% FCS were plated out on Nunclon microtest plates (N1480, well area 0 3 cm²) The medium was replaced after 24 hours The cultures reached confluence in three to four days (as observed under a phase contrast microscope) and were then used for testing The medium was replaced with 200 µl of DMEM containing the isotopes ³H thymidine (TRA 300, 19 Ci/mmol) or ³H-proline (TRA 82, 1 Ci/mmol both from The Radiochemical Centre, Amersham U K) in a concentration of 1 µCi/ml and the IEF fraction of macrophage medium to be tested After incubation for 24 h, the culture medium was pipetted off and the radioactivity in medium collagen measured as described below The cells were detached with 0 25% trypsin collected on glass fibre discs (GF/C, Whatman, Springfield Mill , U K), then washed and counted for incorporated radioactivity (19) in flasks containing 5 ml scintillation fluid (1 g of PPO and 3 3 mg of POPOP in 1 litre of toluene) in a Packard Tri Carb Scintillation Spectrometer (Model 3375, Packard Instrument Co , Inc , Downers Grove, Ill , U S A)

Medium collagen The measurement of radioactive collagen in the fibroblsats culture media after labelling with proline was carried out according to the micromethod of *Jalkanen et al* (in preparation) The pepsinresistant helical sequences of secreted collagen molecules were precipitated with perchloric acid on to glass fibre discs (GF/B Whatman) held in a modified Swinnex 13 membrane holder (Millipore Corp) washed thoroughly by water suction and counted in Tergitol-POP scintillation fluid (15 PPO 50 mg POPOP 600 ml Tergitol® and 400 ml toluene) in the Packard Scintillation Spectrometer

Ribonuclease The enzyme reaction and the precipitation of RNA are described in detail elsewhere (11) The substrate was mixed yeast RNA (Sigma type II) The increase of 1 absorbance unit at 260 nm (molar extinction coefficient = 11 000) equals the liberation of 90 µmol of nucleotide residues

Protein Protein was measured by the method of *Lowry et al* (15) with bovine serum albumin (Sigma Chemicals Co A 4378 St Louis Mo U S A) as standard

RESULTS

pH-Gradients in Isoelectric Focusing

Linear pH gradients formed in the IEF (Fig 1) The pH-range varied, being widest, from 2 to 10 7, at greatest protein load

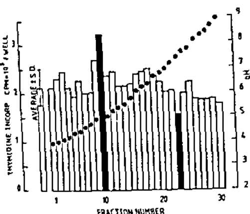


Fig 1 ³H thymidine incorporation into cultured fibroblasts (5th passage) in the presence of the 30 fractions from macrophage culture medium separated by isoelectric focusing Closed circles show the pH values at which the fractions were focused (pH 3 9–8 9) The protein load for focusing was 5 mg Incorporation tests were carried out with fraction volumes of 10 µl per well (*n* = 6) as described in Materials and Methods A typical finding was a rise in thymidine incorporation in the presence of a fraction focusing at pH 4 8 (shaded bar no 10) and a decrease in the presence of a fraction focusing at pH 7 4 (shaded bar no 23) as elaborated in Table 1

from 4 to 8 9, when the amount of protein was small (4–5 mg) or was absent (Fig 1)

Ampholine® is reportedly non-cytotoxic (22) and does not alter the biological activities of proteins We tested the IEF fractions of plain Ampholine on cultured granuloma fibroblasts and found no change in the uptake of ³H-thymide or ³H-proline

Incorporation Tests

All 30 IEF fractions were first assayed for their effect on thymidine incorporation into fibroblasts and for proline incorporation into cells and medium collagen Two fractions were considered of interest because of their effect on thymidine incorporation (shaded bars in Fig 1) and a third for its effect on proline incorporation (see Fig 3)

A fraction (no 23 in Fig 1), located at pH-range 7 3–7 6 (17–17 5 cm from the anode), significantly inhibited *³H-thymidine incorporation* into fibroblasts (Table 1) Moreover, the degree of inhibition depended on the concentration used (Fig 2) An increase in concentration from 2 5 to 20 µl in 200 µl of DMEM decreased the incorporated radioactivity from 91 6 to 30 6%, relative to the level recorded for DMEM alone ($r = -0\ 73$, $p < 0\ 001$) Proline incorporation into both cells and medium collagen remained constant.

A second fraction (no 10 in Fig 1), located at pH 4 4–5 3 (7 5–8 cm from the anode), increased ³H-thymidine incorporation from 117 to 172% over the average of all fractions (Table 1) However, unlike the first fraction, the amount of incorporated ³H thymidine was not dependent on the concentration of fraction 10 over 10 µl Neither had this fraction any obvious effect on proline incorporation

A fraction focusing at pH 10 2–10 7 stimulated *³H-proline incorporation* into the cells and medium

TABLE 1 *³H Thymidine Incorporation into Cultured Fibroblasts in the Presence of Two IEF Fractions from Macrophage Culture Medium*

Run of IEF	Number of assay	Sample volume/well µl	Number of wells	Inhibitory fraction (no 23) Incorporated activity (%)	Stimulatory fraction (no 10) Incorporated activity (%)
1	1	50	3	77 9	171 6
	2	10	3	85 6	136 5
2	1	50	3	66 9	117 2
	2*	10	6	70 2	148 7
3	1	5	3	83 6	117 1
4	1	20	3	68 7	142 6

The figures show the percentage incorporated radioactivity from the average of all the fractions The differences between the average and the inhibitory ($P < 0\ 001$) or the stimulatory ($P < 0\ 005$) fraction were evaluated by the paired *t* test.

* Presented in Fig 1 to which the numbers of the fraction refer

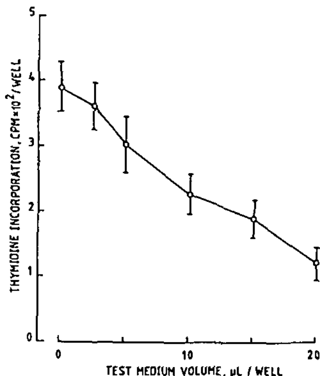


Fig 2 Inhibition of ³H thymidine incorporation into fibroblasts as a function of the concentration of the added IEF-fraction focusing at pH 7 4 (no 23 in Fig 1) The mean ± S E M (n = 6) is given for each concentration

collagen The effect correlated positively with the amount of fraction added (Fig 3) An increase from 2 5 to 20 μl in 200 μl of DMEM increased the ³H-proline uptake into cells by 87% ($r = 0.58$, $p < 0.001$) The radioactivity incorporated into medium collagen was 10–62% higher than with DMEM alone (Fig 3)

Ribonuclease Activity

The most alkaline fraction (pH 10 2–10 7) also exhibited a ribonuclease activity amounting to 30–40% of the original total activity (380 μmol acid soluble nucleotides liberated $ml^{-1}h^{-1}$) in the combined 0–96 h macrophage media The pH optimum for this RNase was 7 0–7 5

DISCUSSION

Dialysis was avoided, and desalting of culture media was carried out by gel filtration to enable the selection of molecules greater than 1800 daltons for IEF The lyophilized IEF-fractions were brought to cell culture conditions by dissolving them in fresh DMEM, and biological assays were carried out without FCS in the culture medium to ensure against interaction between macrophage factors and FCS proteins

Macrophage-derived non-radioactive thymidine has been shown to inhibit ³H-thymidine uptake into fibroblasts (17) This artifact cannot be the cause of inhibition here, however, since low molecular weight substances were removed before IEF by gel filtration

RNase activity was found in the same fraction that stimulated proline incorporation It has been suggested that macrophage derived exudate regulates collagen synthesis in granuloma slices and cell free translation system through an RNase action by means of the degradation of polysomes of fibroblasts (2) It has also been proposed that intracellular RNases and their inhibitors regulate the synthesis of proteins by affecting the stability of polysomes (3, 8, 14) It has recently been shown that RNase is associated also with polyribosomes in fibroblasts of experimental granulation tissue (11) These findings suggest that the intracellular control of protein synthesis of fibroblasts and the macrophage-derived RNase action are connected in some way as yet unknown

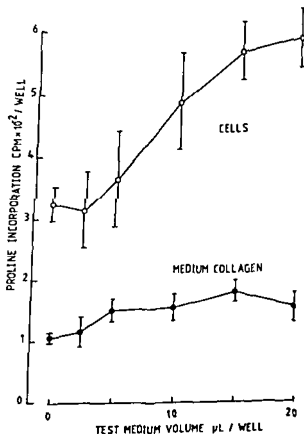


Fig 3 Stimulation of ³H proline incorporation into fibroblasts of 6th passage (–O–O–O–) and medium collagen (–●–●–●–) as a funtion of concentration of a fraction focused at pH 10 4 (mostly alkaline) The protein load for focusing was *15 mg* *The mean* ± S E M (n = 6) is expressed for each concentration

It does not seem reasonable that the RNase activity in the most basic fraction of the macrophage medium would cause a stimulation in the protein synthesis through a degradation of the polysomes in the fibroblasts However, RNase could contribute in the formation of mRNA from its precursors The effect shown in Fig 3 might also be due to other proteins in this basic fraction which is certainly heterogeneous. A number of polycations with high pI have been shown to affect, often biphasically, the enzymatic and metabolic activities of various cells *Wolff & Cook* (24) showed for example, that bovine pancreatic ribunuclease A enhances adenylate cyclase activity of beef thyroid membranes at low concentrations but inhibits it at high concentrations They suggested that the effect was due to charge effects between the polycations and cell membranes The majority of membranes possess a negative net charge (23) and thus probably have a strong affinity for polycationic substances

The findings of the present study support the view that the regulatory functions of macrophages are mediated through different factors which are secreted by the macrophages The particular conditions would favour the production of a specific set of factors

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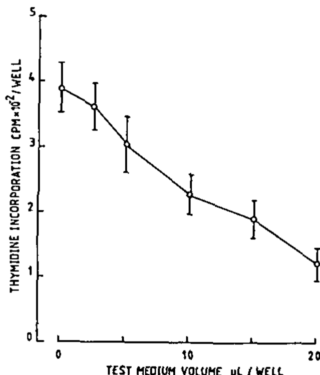


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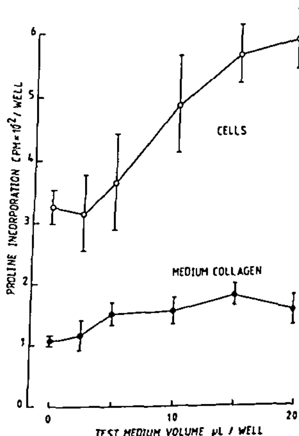


Fig 3 Stimulation of ³H proline incorporation into fibroblasts of 6th passage (–O–O–O–) and medium collagen (–●–●–●–) as a funtion of concentration of a fraction focused at pH 10 4 (mostly alkaline) The protein load for focusing was 15 mg The mean ± S E M (n = 6) is expressed for each concentration

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NON-SPECIFICALLY BOUND IgG AND Fcγ RECEPTORS IN HUMAN MALIGNANT TISSUES

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Wesenberg F Non specifically bound IgG and Fcγ receptors in human malignant tissues Acta path microbiol scand Sect C 87 353–356 1979

Similar amounts of non specifically bound IgG were found in the eluates of the same tissues both after the Fcγ receptor activity (FcRA) had been abolished by disrupting the tissue and when FcRA was intact This indicates that the non specifically bound IgG is either not attached to the FcR or that only the free FcR were abolished No FcRA was detected in the supernatant of the tissue from which the FcRA was abolished thus indicating that the FcRA was destroyed

Key words Human malignant tissues non-specifically bound IgG Fcγ receptors

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We have previously shown that non specifically bound IgG was eluted from five out of thirteen tumours (7 9) but no clear relationship was found between the amount of eluted IgG and the Fcγ receptor activity (FcRA) in corresponding tissue sections (6) The tissue used in these elution experiments was homogenized for 30 s, and the FcRA in sections of tissue sediments was always found to be similar to that in untreated tissue

However disruption in a bacterial press or longer homogenization of the tissue abolished the FcRA in the tissue sediment (6) It was therefore of interest to investigate whether there was any difference in non-specifically bound IgG between eluates of tissue where the FcRA was present and eluates of the same tissue from which the FcRA was abolished Examination was also made as to whether the FcRA was destroyed during a longer period of homogenization

MATERIALS AND METHODS

Tissue

The samples from secondary deposits of two fibrosarcomas and a total of eleven carcinomas from the colon breast, bronchus ovary endometrium cervix and bladder as well as normal liver spleen, kidney lung stomach and skeletal muscle were from the same tissue specimens (marked A M) as those used previously (9)

Disruption of Tissue

Tissue was disrupted in a bacterial press as described previously (6)

Preparation of Extract II

Samples from five of the tumours as well as from

The sediment was then re homogenized for 10 min in four volumes of PBS and centrifuged at 20 000 × g for 20 min The supernatant was concentrated ten times and was called Extract II

Elution of Tissue

3 g tissue disrupted in the bacterial press was eluted by the continuous flow technique as described previously (9) Briefly the tissue was placed between glass fibre filters in a short glass column and washed in a continuous upward flow of PBS containing 0 05% Na azide at 4 °C After a control sample was collected the column was submerged in a waterbath at 56 °C and eluted in the continuous flow of PBS at that temperature Controls and eluates were concentrated to between 1 5 and 2 5 ml (approximately 100 times)

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NON-SPECIFICALLY BOUND IgG AND Fcγ RECEPTORS IN HUMAN MALIGNANT TISSUES

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Wesenberg F Non specifically bound IgG and Fcγ receptors in human malignant tissues Acta path microbiol scand Sect C 87 353–356 1979

Similar amounts of non specifically bound IgG were found in the eluates of the same tissues both after the Fcγ receptor activity (FcRA) had been abolished by disrupting the tissue and when FcRA was intact This indicates that the non specifically bound IgG is either not attached to the FcR or that only the free FcR were abolished No FcRA was detected in the supernatant of the tissue from which the FcRA was abolished thus indicating that the FcRA was destroyed

Key words Human malignant tissues non specifically bound IgG Fcγ receptors

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e have previously shown that non specifically d IgG was eluted from five out of thirteen ours (7 9) but no clear relationship was found een the amount of eluted IgG and the Fcγ ptor activity (FcRA) in corresponding tissue ons (6) The tissue used in these elution riments was homogenized for 30 s and the A in sections of tissue sediments was always d to be similar to that in untreated tissue owever disruption in a bacterial press or longer ogenization of the tissue abolished the FcRA in issue sediment (6) It was therefore of interest vestigate whether there was any difference in specifically bound IgG between eluates of tissue re the FcRA was present and eluates of the tissue from which the FcRA was abolished mination was also made as to whether the A was destroyed during a longer period of ogenization

MATERIALS AND METHODS

he same … as an …

bladder as well as normal liver spleen kidney lung stomach and skeletal muscle were from the same tissue specimens (marked A M) as those used previously (9)

Disruption of Tissue

Tissue was disrupted in a bacterial press as described previously (6)

Preparation of Extract II

The sediment was then re homogenized for 10 min in four volumes of PBS and centrifuged at 20 000 × g for 20 min The supernatant was concentrated ten times and was called Extract II

Elution of Tissue

3 g tissue disrupted in the bacterial press was eluted by the continuous flow technique as described previously (9) Briefly the tissue was placed between glass fibre filters in a short glass column and washed in a continuous upward flow of PBS

Erythrocytes

Erythrocytes of sheep rabbit and ox were obtained and handled as described previously (8) After washing they were packed at 1 000 × *g* for 10 min (100 per cent)

Agglutinins to Rabbit Erythrocytes

The indirect haemagglutination (antiglobulin) titre of eluates tested against rabbit erythrocytes was recorded as described previously (8) These agglutinins are identical with the natural IgG antibodies found in normal sera (8) and have been used as marker antibodies for non specifically bound IgG in the tissue (9)

Sera

Antisera to ox and sheep erythrocytes were raised in rabbits by intravenous immunization

FcRA in Tissue Sections

Demonstration of FcRA in sections of untreated tissue and tissue sediments of homogenized or disrupted tissue was performed as described previously (6) The indicator cells (EA) were sheep erythrocytes (E) sensitized with rabbit IgG antibodies (A) in varying amounts expressed as agglutinating units One agglutinating unit is defined as the amount of the highest dilution of A which agglutinates an equal amount of a 1% suspension of E

Treatment with Neuraminidase

Tissue sections were incubated with neuraminidase (Behringwerke Marburg Lahn West Germany) 100 or 25 I E /ml at 37 °C for 1 h and washed for 10 min in PBS

FcRA in Extract II

Air dried Extract II

One drop of Extract II was air dried on a large cover slip and tested for FcRA as described for tissue sections

Rosette inhibition assay Mononuclear cells were separated from heparinized blood of healthy individuals using centrifugation on a Ficoll Isopaque gradient (Lymphoprep Nyegaard & Co Oslo) washed twice and resuspended to 3 × 10^6 cells/ml in PBS One drop of a suspension of ox erythrocytes sensitized with one agglutinating unit of rabbit IgG antibodies (EA_{ox}) was mixed with one drop of Extract II and incubated for 30 min at approx 20 °C Incubation with PBS served as control One drop of the mononuclear cell suspension was mixed with the suspension and incubated further for 6 h at the same temperature The EA rosette forming cells were then counted in a counting chamber

Agglutination of EA To a twofold dilution of Extract II in 0 1 ml volumes of PBS was added 0 1 ml of a 1% suspension of E sensitized with one half agglutinating unit of A The mixtures were left for 30 min at approx 20 °C The tubes were centrifuged at 1000 × *g* for 30 s the erythrocytes were suspended gently and the agglutination was recorded macroscopically

Concentration

Extract II controls and eluates were concentrated using Amicon UM 05 Diaflo filters

Quantification of IgG and Albumin

IgG in Extract II and eluates and albumin in eluates was quantified using single radial immunodiffusion technique as previously described (9)

RESULTS

Eluates of Malignant Tissue

The main purpose of these experiments was to compare the results obtained previously (9) using eluates made of homogenized tissue where the FcRA was intact with eluates made from samples of the same tissue but where the FcRA was abolished All the eluates of malignant tissue from which the FcRA was abolished contained IgG The amount of IgG eluted was calculated and plotted against the amount obtained using homogenized tissue As shown in Fig 1, the amounts eluted corresponded fairly well although three tumours (E H and L) showed some divergent results From tumour E somewhat more IgG was eluted from the tissue disrupted in the bacterial press and from tumours H and L somewhat more IgG was eluted from the homogenized tissue

Agglutinins to rabbit erythrocytes were detected in the eluates of the four tumours studied previously using homogenized tissue (9) and the ratio of titre of agglutinins to concentration of IgG in the eluates was also similar to that obtained previously (9)

Elution of Normal Tissue

For control purposes normal tissue was disrupted in the bacterial press and eluted Eluates of kidney from younger individuals (aged 20–30

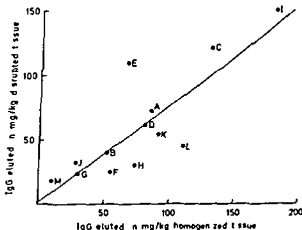


Fig 1 Amount of IgG eluted from homogenized tissue (tumour A–M) plotted against the amount eluted from tissue disrupted in a bacterial press Equation of the regression line y = 0 75x + 3 32 correlation coefficient 0 817 (p <0 001)

TABLE 1 *Adsorption of EA[a)] to Sections of Tissue Sediments from a Carcinoma of the Colon before and after Treatment with Neuraminidase*

Tissue preparations	Neuraminidase[b)]	Agglutinating units of EA				
		2	1	1/2	1/4	1/8
Untreated	–	+ + +	+ +	+	–	–
"	+	+ + +	+ + +	+ +	+	–
Homogenized for 60 s and washed 10 times	–	+ + +	+ +	+	–	–
"	+	+ + +	+ + +	+ +	+	–
Homogenized for 10 min	–	–	–	–	–	–
"	+	–	–	–	–	–

a) Sheep erythrocytes (E) sensitized with varying amounts of rabbit antibodies (A)

b) – before treatment with neuraminidase

+ after treatment with neuraminidase

years) and liver and spleen from individuals of various ages (20–60 years) contained no detectable IgG Eluates of kidney from older individuals (aged 50–60 years) and lung, stomach and muscle from individuals of various ages contained IgG, and some also contained agglutinins to rabbit erythrocytes Where the agglutinins were detected in the eluates the ratio of agglutinins to IgG was similar to that found previously in extracts and eluates of homogenized tissue (9) The amount of IgG eluted from disrupted tissue corresponded to the amount eluted from the homogenized tissue

Ratio of Albumin to IgG

All the eluates contained measurable amounts of albumin The ratio of concentration of albumin to concentration of IgG was therefore calculated where IgG could be measured The results obtained were compared to those obtained using extracts and eluates of the same tissues (7) The ratio found in eluates of all the normal and three of the malignant tissues was similar to that found in extracts and eluates of the homogenized tissues The other malignant tissues showed a reduced ratio as compared to the extract and the reduction obtained was similar to that obtained when eluates of homogenized tissue were used

Extract II

In order to investigate whether the FcRA was destroyed during longer homogenization or whether the FcRA could be detected in the extract, washed tissue sediment with demonstrable FcRA was homogenized until no FcRA could be demonstrated in the sections of the sediment The concentrated extract (Extract II) contained either no IgG or only trace amounts EA sensitized with from one-eighth to two agglutinating units did not adsorb to Extract II [illegible]

of A

EA sensitized with from one-eighth to two agglutinating units did not adsorb to sections of tissue homogenized for 10 min Incubation of these sections with Extract II or washing the sections in PBS at 4 °, 20 °, 37 °, 45 ° or 56 °C for 30 min did not restore the FcRA There were no differences in the results obtained using malignant tissue or normal liver and spleen

Treatment of Tissue Sections with Neuraminidase

Representative results obtained using neuraminidase are shown in Table 1 Untreated tissue adsorbed EA stronger after treatment with neuraminidase, but no adsorption was visible on the sections of tissue homogenized for 10 min after treatment with neuraminidase

DISCUSSION

In this study, comparison has been made of eluates of sediment of malignant tissue where the FcRA could be demonstrated in sections of the sediment, and eluates of samples of the same tissue from which the FcRA had been abolished The results obtained show that about the same amount of both

total and non specifically bound IgG was eluted from the tissue sediment irrespective of whether or not the FcRA could be demonstrated in the sections of the sediment This could mean that the non specifically bound IgG is not attached to the FcR as indicated previously (6) However another explanation might be that only the free FcR detected by the indicator cells used were abolished from the tissue The FcR that had bound IgG would then not be affected and this IgG could be eluted at 56 °C

FcR have been isolated from lymphoid tissues using different techniques (1–5) Since the FcRA could not be detected in sections of tissue homogenized for 8 min (6) the question therefore arose as to whether the FcRA could be detected in the supernatant (extract) of the tissue after the FcRA was abolished No FcRA was detected in the extract Furthermore treatment of sections of the homogenized tissue from which the FcRA had been abolished with neuraminidase did not reveal any hidden FcRA as was the case when untreated tissue was used Washing of the sections at different temperatures did not reveal any FcRA thus indicating that the FcRA was destroyed However the concentration of FcRA in the Extract II might be too low for detection of FcRA The FcRA in the supernatant might also be blocked though this is less likely since there was no IgG or only trace amounts of IgG in the supernatant Therefore the results obtained show that other methods than those described here must be used for the isolation of FcR in malignant tissue

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Acta path microbiol scand Sect C 87 357-364 1979

ACTIVATION OF THE BACTERICIDAL CAPACITY OF BLOOD GRANULOCYTES

Evaluation of a New Method and the Effect of Levamisole

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Palmblad J & Engstedt L Activation of the bactericidal capacity of blood granulocytes Evalution of a new method and the effect of levamisole Acta path microbiol scand Sect C 87 357-364 1979

In most polymorphonuclear (PMN) bactericidal assays each PMN is given appr 0 5–4 bacteria to kill but it has not been sufficiently shown that this ratio is optimal for the detection of changes in PMN killing functions One PMN incubated with increasing amounts of *Staph aureus* can kill between 45 and 90 colony forming units (CFU) Therefore a new assay giving each PMN a submaximal bacterial amount (i e 30–40 CFU/PMN) was developed This new assay was compared with a standard one (i e using 2-4 CFU/PMN) concerning the effects on PMNs of thermal inactivation and of levamisole Exposure to 46 °C for 3-5 minutes decreased the killing capacity easiest descernable with the standard assay Incubation with levamisole in concentrations ranging between 10^{-6} and 10^{-7} mol/l increased the PMN killing capacity and this was most evident with the modified assay Thus enhancements of PMN killing functions might be detected better with the present modified PMN bactericidal assay whereas impairments are disclosed more readily with the standard assay

Key words Blood granulocytes bactericidal capacity new test method levamisole

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Polymorphonuclear (PMN) granulocyte bactericidal functions are usually studied with *in vitro* assays After incubation of fixed numbers of PMNs and bacteria the number of remaining living bacteria are determined either by the Maaloe technic or by biochemical tests Most conventional tests employ an initial ratio between PMNs and bacteria which is appr 1 to 1 (2 8 25) Although it has not been sufficiently demonstrated that this ratio is optimal for different purposes it has been used widely and apparently successfully for the identification of various defects in PMN killing functions

It has been argued however that it would be easier to detect changes of PMN bactericidal function if the number of bacteria per PMN is increased (10) Recently *Clawson & Repine* (4) suggested a test for the detection of PMN defects where up to 100 bacteria per PMN was employd The usefulness of this »stressed« test has however been debated (3)

In addition to investigations concerning defects of PMN killing functions it seems equally interesting to obtain information about a possibly enhanced bactericidal capacity To »stimulate the phagocytes« (24) is still an important task for antimicrobial therapy Tools for such observations are certainly needed e g in pharmacological research

The aim of this report is to present work on a modification of the Maaloe technic whereby each granulocyte is given approx 30–40 bacteria to kill in vitro (10) The fitness of the modified method has been examined and compared with a standard assay in two experimental models presented here

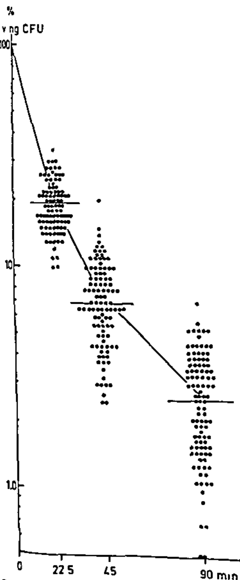
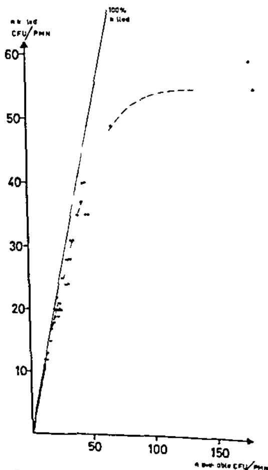


Fig 1 Results from the analyses of PMNs from 103 hea thy donors when ncubated with the »low« bacterial inoculum The med an value is for the 45 minute incubation 7 0% and for the 90 minute 2 6% The reference values (i e the lim ts for the central 95 percent of the material) are for the 45 m nute incubation 2 7 14 8% and for the 90 m nute 0 5–5 4%

The Submaximal Bacterial Concentrat on (the Mod fed Assay)

According to the results in Fig 2 the maximal PMN killing capacity varied considerably To determine this capacity several bacterial d lutions would be needed which would require considerable quantities of granulocytes Also an increased risk of cell rupture has been observed at high bacterial concentrations (4)

Instead therefore a submaximal bacterial con centration was chosen aiming at giving each PMN appr 30–40 CFU to kill With a careful photome tric adjustment it was possible to obtain a standardized bacterial concentration in the test tube of $9\,2 \pm 2\,0 \times 10^{10}$ (mean and S D) CFU/l This is henceforth called the »high« bacterial inoculum (B I) To test whether the bacteria were in the *lag or log phase* and if the serum possessed staphylocidal properties 50 controls were run for 90 minutes with only bacteria and serum These yielded for the »low« and »high« bacterial concentrations in mean (± SE) 131 ± 9 and 107 ± 5 percent of the initial counts respectively Thus no appreciable bacterial



MATERIAL AND METHODS

Subjects

Donors of PMNs were 103 healthy blood bank donors and members of the laboratory staff Their health was assessed by the routine procedure employed by Stockholm Blood Centre None was pregnant or on a drug regimen

The Standard and Modified PMN bactericidal assays

Basically the PMN bactericidal capacity has been investigated as described previously (11) but with lower PMN concentration In short dextran sedimented twice washed granulocytes obtained from heparinized venous blood were mixed with pooled serum and suspensions of *Staph aureus* phage type 52A/79 group I grown over night in broth and being in the lagphase The bacteria had been washed twice in 0 45% saline and then resuspended The desired bacterial concentration has been adjusted with saline according to its optical density in a Beckman B spectrophotometer (11) The concentration in the test tube of serum was 10% and of granulocytes 2 5 × 10^9/l (range 2 25–2 75) and only band and segmented forms were counted The concentration of bacteria was 1 0 × 10^{10}/l colony forming units (CFU) in the standard assay and in the modified assay as described below The tubes were incubated at 37 °C in duplicate and samples were removed after 22 5 45 and 90 minutes of incubation for quantitation of viable CFU by the pour plate method The results are given in terms of mean living CFU in the duplicates as a percentage of the initial counts

Thermal Inactivation

To create a reproducible and partial defect of PMN bactericidal capacity affecting all cells a PMN suspension from a single subject was incubated in a 46 °C water bath for 1 to 5 minutes after which bacteria and serum were added The bactericidal capacity was then assessed with the standard and modified assays in order to evaluate their ability to detect the impairment Untreated PMNs from the same subject served as controls The difference in the percentages of living bacteria from the controls formed the basis for the statistical analyses and the mean values of 45 and 90 min incubation periods are depicted in Fig 5

Levamisole

The antihelmintic drug levamisole (L 2 3 5 6 tetra hydro 6 phenyl midazo thiazole hydrochloride mol wt appr 241 kindly provided by Jansen Division Leo Helsingborg Sweden) was used to see whether a possible stimulation could be observed with either assay The drug was dissolved in Hanks balanced salt solution containing 0 1 % gelatin (HBSS) to a stock concentration (10^{-2}mol/l) from which final dilutions were made The stock solution was kept at + 4 °C for up to a week The PMNs were incubated with levamisole (10^{-4}–10^{-8} mol/l) at room temperature for 30 minutes prior to addition of serum and bacteria To the control tubes equal volumes of HBSS were added As for the thermal inactivation the difference in the CFU percentage values from the controls was analyzed statistically and the mean values of the 45 and 90 min incubation are depicted in Fig 6

Statistical Analyses

These were performed using the Wilcoxon s rank sum test for the evaluation of changes within one group of experiments and the Mann Whitney rank sum test for evaluation of changes between groups When analyzing whether the standard or the modified assay was better for the detection of PMN changes secondary to heat and levamisole treatment the 45 and 90 minute incubation values were used together as a basis for the calculation The goodness of fit of the percentages living CFU at 90 minutes incubation was tested for normality of distribution by means of the Kolmogorov Smirnov test for both the original percentage values and after logarithmic transformation

RESULTS

The Standard Assay

The results from the analyses of the 103 healthy subjects are given in Fig 1 For conveniance this has been drawn in a logarithmic scale However since a logarithmic transformation of the percentages did not improve the normality of distribution of results ($p > 0.05$) all statistical analyses were performed on the original percentage values The day to-day variation for a single subject is given in Table 1 The error of the method is 16% when calculated from 20 double determinations by the formula

$$\sqrt{\frac{\Sigma d^2}{2n}}$$

The variation (mean ± SD) of the standard bacterial inoculum (referred to hereafter as the ›low‹ BI) was 1 05 ± 0 37 × 10^{10} CFU/l Thus each granulocyte provided with a mean of 3–4 CFU

Maximal PMN Killing Capacity

As described above desired concentrations of CFU could be obtained after a photometrical adjustment The final test tube concentrations were accordingly varied from a maximum of 2 5 × 10^{11} CFU/l with several serial dilutions down to the »low« BI i e appr 1 × 10^{10} CFU/l In this manner each granulocyte from a single donor could initially be provided with from 200 to 2–4 CFU The number of CFUs remaining viable after 90 minutes of incubation was calculated and the results from 105 determinations are given in Fig 2 The maximal killing capacity varied but was generally between 45 and 90 CFU/PMN

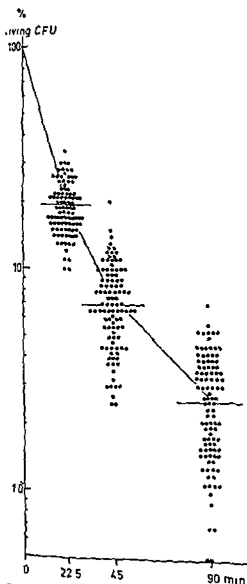


Fig. 1. Results from the analyses of PMNs from 103 healthy donors when incubated with the »low« bacterial inoculum. The median value is for the 45 minute incubation 7.0% and for the 90 minute 2.6%. The reference values (i.e. the limits for the central 95 percent of the material) are: for the 45 minute incubation 2.7–14.8% and for the 90 minute 0.5–5.4%.

The Submaximal Bacterial Concentration (the Modified Assay)

According to the results in Fig. 2 the maximal PMN killing capacity varied considerably. To determine this capacity, several bacterial dilutions would be needed, which would require considerable quantities of granulocytes. Also an increased risk of cell rupture has been observed at high bacterial concentrations (4).

Instead, therefore, a submaximal bacterial concentration was chosen, aiming at giving each PMN appr. 30–40 CFU to kill. With a careful photometric adjustment it was possible to obtain a standardized bacterial concentration in the test tube of $9.2 \pm 2.0 \times 10^{10}$ (mean and S.D.) CFU/l. This is henceforth called the »high« bacterial inoculum (B.I.). To test whether the bacteria were in the *lag or log phase* and if the serum possessed staphylocidal properties, 50 controls were run for 90 minutes with only bacteria and serum. These yielded for the »low« and »high« bacterial concentrations in mean (± SE) 131 ± 9 and 107 ± 5 percent of the initial counts, respectively. Thus no appreciable bacterial

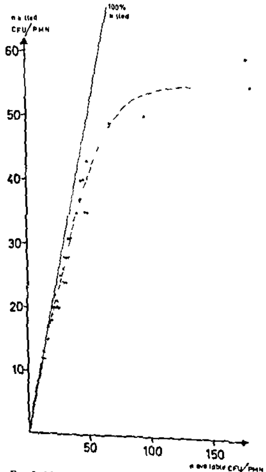


Fig. 2. Maximal killing capacity of human PMNs. The initial CFU/PMN ratio is given on the horizontal axis and the 90 minute incubation ratio on the vertical axis.

TABLE 1 *PMN Bacterial Capacity Day to day Variation in the Percentage Living CFU at 90 Min Incubation*

Donor		% living CFU at 90 min Day 1	Day 2	Day 3	Day 4	Mean ± SE
A	low B I	3 6	3 6	2 2		3 1 ± 0 5
	high B I	16 6	20 4	15 9		17 6 ± 1 4
B	low B I	4 2	5 5	5 5	5 4	5 2 ± 0 3
	high B I	16 6	10 2	15 9	13 5	14 0 ± 1 4
C	low B I	4 2	2 4	2 4		3 0 ± 0 6
	high B I	23 6	12 7	18 7		18 3 ± 3 2
D	low B I	2 5	4 9	1 3	1 8	2 8 ± 0 5
	high B I	13 8	27 5	13 9	20 6	19 0 ± 3 3
E	low B I	1 3	2 4	2 8	3 8	2 6 ± 0 5
	high B I	16 2	11 1	13 4	10 9	12 9 ± 1 2
F	low B I	1 7	1 3	1 9		1 6 ± 0 2
	high B I	15 2	11 7	19 3		15 4 ± 2 2

growth occurred during the incubation Serum is essential for the killing of this *Staph aureus* strain as evidenced by the findings when serum was replaced by HBSS in the test tube With the »low« BI the 90 minute incubation results were in mean (± SE) 85 8 ± 2 2 percent and with the »high« BI 75 8 ± 9 1 The effect of the *mixing procedure* i e 20 end over end rotations per minute in a Heto Rotomixer was compared with no mixing at all With the latter only marginal killing was seen the 90 min incubation values were in mean 87 4 and 75 0 percent with the »low« and »high« B I respectively With PMNs from the *103 donors* the results obtained with this modified PMN bactericidal assay are depicted in Fig 3 As with the standard assay the results obtained with the modified assay were more normally distributed when the original percentages were used compared with their logarithmic transformations ($p > 0\ 05$) Hence the former were used for statistical purposes

The day to day variation for a single subject is given in Tabel 1

The *error of the method* calculated as described above is 8 1 %

Whether *sex or age* were associated with changes in PMN bactericidal functions has been determined with the standard as well as the modified assays Thirty one males and the same number of females showed the following mean percentages (± SE) after 90 minutes incubation »low« B I 2 7 ± 0 3 and 2 5 ± 0 2 respectively »high« B I 17 5 ± 0 7 and 17 4 ± 0 9 respectively Thus no differences between the sexes could be found in this respect Age between 20 and 90 years has previously been shown not to be associated with a change in PMN killing functions with either method (14) The effect of a previous intake of *food* has been determined A breakfast may give a tendency to an enhancement of the PMN killing capacity with »high« B I but a comparison with the day to-day variation shows no major difference (Fig 4)

Thermal Inactivation

With increasing periods of exposure to 46 °C PMN bactericidal capacity decreased Only small deviations from the control values were noted with either B I if the exposure was 3 minutes or less In the 4 and 5 minute experiments pronounced and significant impairments were noted with the standard assay (»low« B I) while the results with the modified assay (»high« B I) only showed significant changes in the 5 minute samples (Fig 5) Thus a minor reduction of the PMN bactericidal capacity was disclosed better by the standard than the modified assay

Levamisole

As shown in Fig 6 the modified assay exhibited significant reductions of the percentages of living CFU when incubated with levamisole concentrations of 10^{-6} and 10^{-7} mol/l while the standard

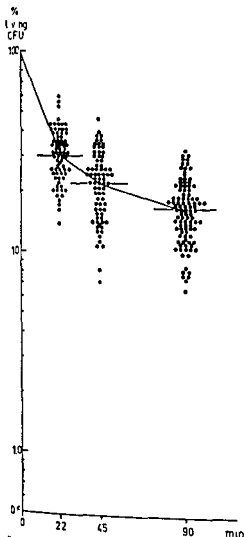


Fig 3 Results from the analyses of PMNs from 103 healthy donors when incubated with the »high« bacterial inoculum. The median value is for the 45 minute incubation 22.9% and for the 90 minute 16.6%. The reference values (i.e. the limits for the central 95 percent of the material) are for the 45 minute incubation 8.3–36.8% and for the 90 minute 7.7–29.7%.

assay (»low« B I) only showed a small but significant difference from the controls at the 10^{-6} mol/l level where also a significant difference was observed between the »high« and »low« B I.

DISCUSSION

The present findings indicate that human PMN granulocytes are able to kill at most, 45–90 *Staph aureus in vitro*. This capacity can be used in a modified bactericidal assay where each PMN is given 30–40 bacteria and which possesses a reasonable precision. Based on the experiments presented here and elsewhere (vide infra) we suggest that compared with a standard assay this modified assay will primarily enhance the ability to detect a stimulated but not a decreased PMN bactericidal capacity. With this modified assay evidence is

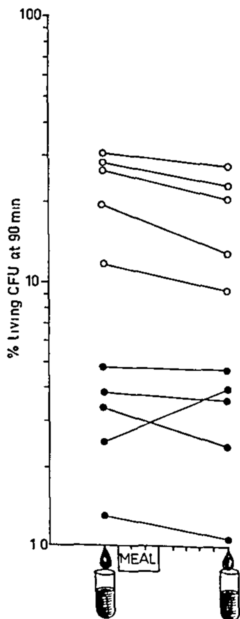


Fig 4 The influence of a meal as described in text on 5 subjects fasted over night. ●—● »low« BI, O–O »high« BI

TABLE 1 *PMN Bacterial Capacity Day-to-day Variation in the Percentage Living CFU at 90 Min Incubation*

Donor		% living CFU at 90 min Day 1	Day 2	Day 3	Day 4	Mean ± SE
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B	low B I	4 2	5 5	5 5	5 4	5 2 ± 0 3
	high B I	16 6	10 2	15 9	13 5	14 0 ± 1 4
C	low B I	4 2	2 4	2 4		3 0 ± 0 6
	high B I	23 6	12 7	18 7		18 3 ± 3 2
D	low B I	2 5	4 9	1 3	1 8	2 8 ± 0 5
	high B I	13 8	27 5	13 9	20 6	19 0 ± 3 3
E	low B I	1 3	2 4	2 8	3 8	2 6 ± 0 5
	high B I	16 2	11 1	13 4	10 9	12 9 ± 1 2
F	low B I	1 7	1 3	1 9		1 6 ± 0 2
	high B I	15 2	11 7	19 3		15 4 ± 2 2

growth occurred during the incubation Serum is essential for the killing of this *Staph aureus* strain as evidenced by the findings when serum was replaced by HBSS in the test tube With the »low« BI the 90 minute incubation results were in mean (± SE) 85 8 ± 2 2 percent, and with the »high« BI 75 8 ± 9 1 The effect of the *mixing procedure* i e 20 end over-end rotations per minute in a Heto Rotomixer was compared with no mixing at all With the latter only marginal killing was seen the 90 min incubation values were in mean 87 4 and 75 0 percent with the »low« and »high« B I respectively With PMNs from the *103 donors* the results obtained with this modified PMN bactericidal assay are depicted in Fig 3 As with the standard assay the results obtained with the mod ified assay were more normally distributed when the original percentages were used compared with their logarithmic transformations ($p > 0 05$) Hence the former were used for statistical purposes

The day-to-day variation for a single subject is given in Tabel I

The *error of the method* calculated as described above is 8 1 %

Whether *sex or age* were associated with changes in PMN bactericidal functions has been determined with the standard as well as the modified assays Thirtyone males and the same number of females showed the following mean percentages (± SE) after 90 minutes incubation »low« B I 2 7 ± 0 3 and 2 5 ± 0 2, respectively »high« B I 17 5 ± 0 7 and 17 4 ± 0 9 respectively Thus no differences between the sexes could be found in this respect Age between 20 and 90 years has previously been shown not to be associated with a change in PMN killing functions with either method (14) The effect of a previous intake of *food* has been determined A breakfast may give a tendency to an enhancement of the PMN killing capacity with »high« B I, but a comparison with the day-to day variation shows no major difference (Fig 4)

Thermal Inactivation

With increasing periods of exposure to 46 °C PMN bactericidal capacity decreased Only small deviations from the control values were noted with either B I if the exposure was 3 minutes or less In the 4 and 5 minute experiments pronounced and significant impairments were noted with the standard assay (»low« B I) while the results with the modified assay (»high« B I) only showed significant changes in the 5 minute samples (Fig 5) Thus a minor reduction of the PMN bactericidal capacity was disclosed better by the standard than the modified assay

Levamisole

As shown in Fig 6 the modified assay exhibited significant reductions of the percentages of living CFU when incubated with levamisole concentrations of 10^{-6} and 10^{-7} mol/l while the standard

modified bactericidal assay where each PMN is given 30–40 bacteria and which possesses a reasonable precision. Based on the experiments presented here and elsewhere (vide infra) we suggest that compared with a standard assay this modified assay will primarily enhance the ability to detect a stimulated but not a decreased PMN bactericidal capacity. With this modified assay evidence is

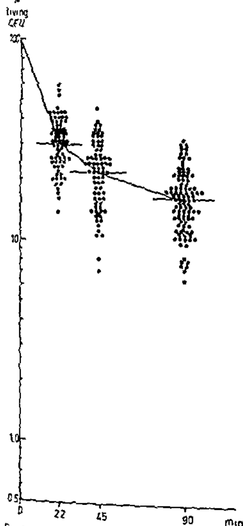


Fig. 3. Results from the analyses of PMNs from 103 healthy donors when incubated with the »high« bacterial inoculum. The median value is for the 45 minute incubation 22.9% and for the 90 minute 16.6%. The reference values (i.e. the limits for the central 95 percent of the material) are for the 45 minute incubation 8.3–36.8% and for the 90 minute 7.7–29.7%.

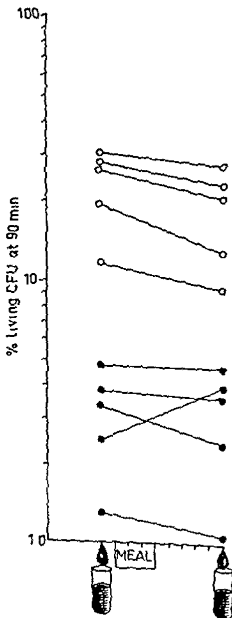


Fig. 4. The influence of a meal as described in text on 5 subjects fasted over night. ●—● »low« BI. O–O »high« BI.

assay (»low« B.I.) only showed a small but significant difference from the controls at the 10⁻⁶ mol/l level where also a significant difference was observed between the »high« and »low« B.I.

DISCUSSION

The present findings indicate that human PMN granulocytes are able to kill at most 45–90 *Staph. aureus* in ... city can be used in a

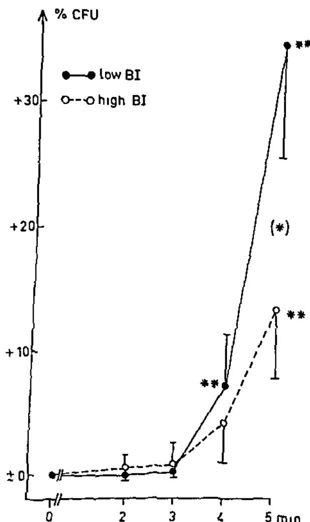


Fig 5 Effects on the bactericidal capacity of various periods of thermal inactivation of the PMNs Means and SE for the differences from the non exposed cells
●—● low BI ○—○ high BI Statistical significance of differences are given from untreated samples and within brackets for the differences between the high and low BI * $P < 0.05$ *(*) $P < 0.02$ ** $P < 0.01$

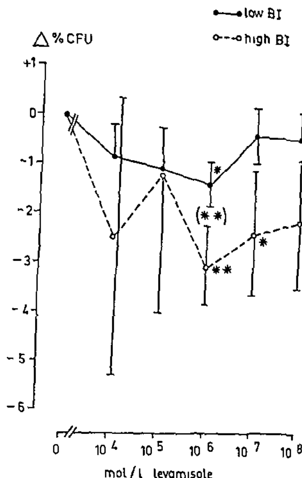


Fig 6 The effects on PMN bactericidal capacity of levamisole Means and SE values for the difference from the controls Symbols as in Fig 4

presented here that the antihelmintic drug levamisole might stimulate PMN killing functions *in vitro* in addition to the enhancements of PMN chemotaxis and engulfment reported by others (9 26 cf however 21)

The maximal PMN killing capacity has previously been estimated to be appr 60 colony forming units (CFU) per PMN (4 10) The present findings are in general agreement with these studies but emphasize the difficulty to design an *in vitro* test which measures this adequately without creating artefacts For instance if 100 bacteria or more are given to each PMN granulocyte structure changes and cell rupture were observed after a short period of incubation with tumbling of the test tubes necessitating a termination of tumbling already after 20 minutes (4) However since a considerable degree of killing takes place between both 20 and 45 and 45 and 90 minutes of incubation with the high as well as the low bacterial inoculum (BI) valuable informations might be lost when incubation is stopped after 20 minutes Instead it is suggested that the submaximal bacterial inoculum used here might possibly be a less cell damaging exposure since the killing is continuous during the whole incubation time The variation in the end results is also considerably less than reported with the stress test (4)

The day to day variation for one subject is small as presented here (Table I) This however presupposes thorough control of e g the granulocyte concentration in the test tube since such variations are of greater importance for the results of the bactericidal assay than the bacterial concentrations (*Palmblad et al* to be pub

tions are taken it will also be found that the between subject variation is rather small (Fig 1 & 2) Thus there is no need to use the neutrophil bactericidal index by *Alexander et al* (1) or the leukocyte bactericidal indices proposed by *Hoffman & Bullock* (7) when patients are compared with controls The latter moreover employ the expression log percent to describe the distribution of normal results As found here a logarithmic transformation did not improve the normality of distribution of results and consequently it has not been used Thus with the present methods a simpler way of comparing differences between groups can be used (cf 6) However even with the linear distribution some unilateral distortion remains which makes it unsuitable to use the standard deviation as a basis for establishing the reference values Hence for this purpose we have chosen to give the limits as those for the central 95 percent around the median value

It has been suggested that an increase in the amount of bacteria that each PMN is challenged with could better identify small defects of PMN killing functions (4) In subsequent studies these high bacterial concentrations did not in fact reveal any new defects which could not be assessed with the traditional assay (19 20 21) In the present study the high BI assay did not detect PMN defects with increased sensitivity compared with the standard assay Instead the latter did so better when the effects of thermal inactivation of PMNs were investigated whereas the modified assay detected levamisole associated enhancements better

The usefulness of the standard and modified assays for detecting PMN ...

... surgery the standard assay showed decreases of the bactericidal capacity more clearly than the modified but on the other hand the modified revealed postoperative enhancements better (16 22) In another study of patients operated with small bowel shunts for morbid obesity similar conclusions were made (17) These findings are also supported by unpublished observations from studies on the ...

... method (14 15) The ability of the modified assay to detect stimulation of PMN bactericidal mechanisms is further exemplified by the study of the influence of levamisole This drug has previously been shown to stimulate in vitro granulocyte chemotaxis (26) the uptake of yeast cells (9) and to restore depressions of these functions in patients with decreased host defences (5 18 23 26) However no stimulation of PMN bactericidal activity was found when a higher *in vitro* levamisole concentration (100 μg/ml i e appr 4 × 10^{-4} mol/l) was used than in the other studies (21) The concentration of levamisole found to give maximal stimulation of PMN chemotaxis (26) was similar to what has been found here (10^{-7} mol/l) but is somewhat less then that yielding maximal stimulation of particle uptake (i e 4 × 10^{-6} mol/l) (9) Interestingly the stimulation occurred over a rather broad range of concentrations in both previous studies as it did here

The mechanism by which levamisole exerts its stimulation is not known Suggestion have been made that it is through some effect on plasma increasing the opsonization which could explain the wide range of efficient concentrations (9) Both the rate of attachement (3) and uptake of bacteria and changes in the directly bactericidal mechanisms may be important for the rate and degree of killing

Based on present studies it is suggested that the modified assay might be used alongside the standard assay thereby securing optimal coverage of both possible impairments and enhancements of PMN bactericidal functions

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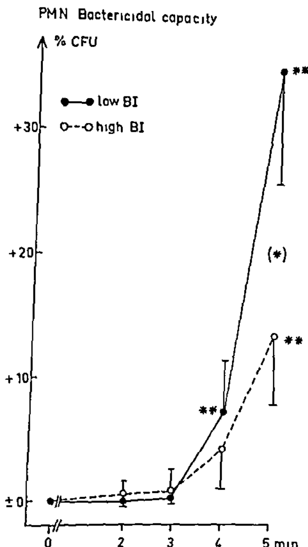


Fig 5 Effects on the bactericidal capacity of various periods of thermal inactivation of the PMNs Means and SE for the differences from the non exposed cells
●—● low BI O—O high BI Statistical signifiance of differences are given from untreated samples and within brackets for the differences between the high and low BI * P < 0 05 *(*) = P < 0 02 ** = P < 0 01

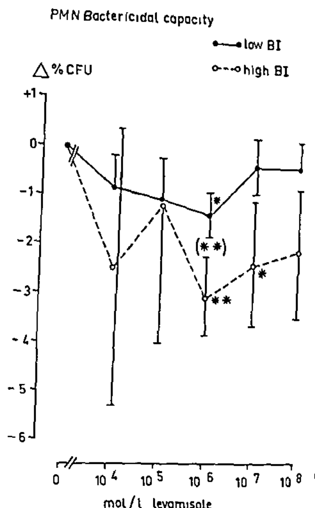


Fig 6 The effects on PMN bactericidal capacity of levamisole Means and SE values for the difference from the controls Symbols as in Fig 4

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ANTICOMPLEMENTARY ACTIVITY IN DIFFUSE AND NODULAR GOITERS

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Feldt Rasmussen U Bech K Date J Thyme S Hannover Larsen J & Nielsen H Anticomplementary activity in diffuse and nodular goiters Acta path microbiol scand Sect C 87 365-370 1979

The occurrence of anticomplementary activity and its correlation to serum thyroglobulin was investigated in 71 patients with thyroid diseases and 63 age and sex matched control subjects The patients which were subgrouped according to thyroid function and characteristics of the goiter were examined at the time of diagnosis The anticomplementary activity was measured by a complement consumption (CC) assay Sera from patients with Graves disease and nontoxic diffuse goiter showed stronger activity than sera from patients with nontoxic goiter Seventeen of the patients and one of the controls were positive in the CC-assay The percentage hemoglobin release in this assay was normally distributed using control sera but not for the patient group There was no correlation either between CC activity and serum concentrations of thyroglobulin or CC activity and antibodies to the O antigen of *Yersinia enterocolitica* serotype 3 thyroid cytoplasma thyroglobulin nuclear factors streptolysin O streptococ hyaluronidase and parietal cells respectively However correlation between the levels of thyroid stimulating immunoglobulins and CC activity was noted (Rho = 0 511 P <0 05) which suggests that these immunoglobulins also are present as immune complexes Thyroglobulin – antithyroglobulin complexes preformed *in vitro* at high thyroglobulin concentration gave negative results in the CC assay

Key words Complement consumption assay thyroid diseases thyroglobulin thyroglobulin autoantibodies thyroid stimulating antibodies

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Both humoral and cell mediated immune reac ons are implicated in thyroid diseases especially Graves and Hashimoto s diseases as reviewed by *Volpe et al* (1974) and *Allison* (1976) A variety of hyroid autoantibodies are found (*Hall* 1962) and *in itro* inhibition of leucocyte migration in response to hyroid homogenate (*Lamki et al* 1973 *Wartenberg et al* 1973 *Bech et al* 1978) and thyroglobulin Tg) (*Delespesse et al* 1973) has been described

The presence of circulating immune complexes (IC) indicated by inhibition of K-cell cytotoxic activity (*Calder et al* 1976) and anticomplementary activity (*Calder et al* 1974) has been reported in autoimmune thyroid disease Further more serum sickness likely caused by complexes composed of thyroglobulin (Tg) – antithyroglobulin (TgAb) has been observed following therapy in Graves disease (*Ploth et al* 1978)

The purpose of this investigation was to study the occurrence of anticomplementary activity in diffuse and nodular goiter and to examine whether this activity correlates to the levels of Tg TgAb thyroid

This work was presented in part at the 4th European Immunology Meeting in Budapest April 1978

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berg 1976). The ratios between Tg and TgAb ranged from ten fold antigen excess to ten fold antibody excess in terms of binding sites. Control tubes of EN, Tg and NS were included and all tubes were incubated for one hour at 37 °C and at 4 °C overnight. After further incubation at 56 °C for 30 minutes they were all run in the CC assay.

RESULTS

In the patients an increased level of anticomplementary activity (CC) was demonstrated as compared with controls. Seventeen patients were CC positive in contrast to only one control subject ($P < 0.001$, Table 2).

The distribution of CC activity of patients and controls were compared (Fig. 1A) and both median and upper range were higher in patients than in controls. Plotting the cumulated frequency on a probit scale yielded a straight line for the controls while this could not be seen in the group with thyroid disorders (Fig. 1B).

Comparing the subgroups of patients with their respective controls (Table 1) no difference in respect to sex and age was demonstrable. The CC activities differed most markedly when comparing nontoxic diffuse goiter with the relevant control group ($P < 0.02$, Fig. 2). In nontoxic nodular goiter and Graves' disease the difference was of the same magnitude compared to the control group ($P < 0.05$) but the median value was higher for the group with Graves' disease. Only few patients with toxic adenomas were included and no significantly increased level of CC activity was demonstrable.

There was no demonstrable correlation between CC-activity and antibody levels to thyroid cytoplasma, the O antigen of *Yersinia enterocolitica* serotype 3 as well as ANF, ASH, AST and parietal cell antibodies, all measured in the same patient sera.

TABLE 2. *Anticomplementary Activity Measured by a Complement Consumption Assay in Thyroid Disease*

	Patients	Controls
CC positive[a]	17[b]	1[b]
CC negative	54	62
Total	71	63

[a] The level of positivity was set by the mean per cent inhibition of hemoglobin release + 2 SD of controls.
[b] $P < 0.001$ (χ^2-test).

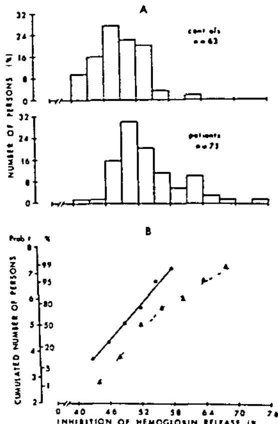


Fig. 1 A. Distribution of results from the CC test in patients with thyroid disease and controls. B. Cumulated distribution of the same results as in Fig. 1A. (Probit/linear scale). Each point indicates the percentage of persons (ordinate) with a CC test result below the value read off the abscissa. ● controls (n = 63). ▲ patients (n = 71).

Furthermore, no correlation of activity was demonstrated either to the serum levels of native Tg or TgAb (Table 3). In 18 patients with Graves' disease a significantly positive correlation between TSAb and CC activity was found (Rho = 0.511, $P < 0.05$). TSAb results for all groups are shown in Table 3.

Finally, Tg IC preformed *in vitro* covering antigen antibody ratios in term of binding sites from 5 to 10 times antigen or antibody excess, respectively, were tested in the CC-assay. No CC activity could be demonstrated for any of the ratios.

DISCUSSION

The results of the CC test expressed as per cent inhibition of hemoglobin release were normally distributed in controls, while in patients the

stimulating antibodies (TSAb) or other autoantibodies present in these diseases A preliminary report has been published (*Feldt Rasmussen et al* 1977)

MATERIAL AND METHODS

Patients Seventy one unselected outpatients 8 males and 63 females with thyroid diseases were studied They were subgrouped (Table 1) according to thyroid function as evaluated by serum thyroxine (T_4) serum triiodothyronine (T_3) serum T_3 uptake (T_3 U) serum thyrotropin (TSH) 131 iodine uptake by the thyroid gland and in some patients thyrotropin releasing hormone test The thyroid gland was examined by palpation and Tc99 scintigraphy to classify the goiter as either diffuse nodular or a solitary adenoma Graves disease was defined as diffuse goiter and hyperthyroidism often associated with exophtalmos and/or pretibial myxoedema All the patients were investigated at the time of diagnosis before treatment was started The age and sex distributions of the subgroups are seen in Table 1

Controls The control group comprised 63 healthy nonhospitalized persons matched according to sex and age to the patient group (Table 1) Neither before nor at the time of investigation did they have any clinical or

with iodine contrast dyes that might disturb the thyroid

results were expressed as per cent inhibition of hemoglobin release Patient sera with inhibiting activity exceeding the mean + 2 SD of results obtained with se from 63 age and sex matched control subjects we considered positive in the CC test

Thyroid function tests Serum T_3 U was performed described by *Hansen* (1964) serum T_4 by a modificatio of Murphy s method (*Siersbæk Nielsen* 1967) and seru T_3 and TSH by radioimmunoassays (*Skovsted* 197 *Weeke & Orskov* 1973) Serum Tg and TgAb we measured as previously described (*Feldt Rasmussen* *al* 1979a) Thyroid stimulating antibodies (TSAb) we measured by stimulation of adenylate cyclase activity i human thyroid homogenates and expressed as per cent c reference activity as previously described (*Bech & Mad sen* 1978) Antibodies to *Yersinia enterocolitica* serotyp 3 were determined by an agglutination technique an antibodies to streptolysin O (AST) and streptoco hyaluronidase (ASH) were measured as described b *Larsen* (1976) Thyroid microsomal antibodies an antinuclear factor were determined by immunofluore scence technique (*Weller & Coons* 1954)

Serum specimens were stored up to 6 months at −2(°C and patient and control sera were run in the sam assay series

Statistical evaluation Data were analysed for statisti cal significance using Mann Whitney s test for indepen dent samples and χ^2 test The Spearmann s rank correlation (Rho) was used also

Thyroglobulin antithyroglobulin immune complexes (Tg IC) in vitro Highly purified 19 S Tg (*Feldt Rasmus sen* 1978) at a concentration of 20 mg/ml was mixed with serial dilutions of a serum from a patient (FN) with Graves disease and a TgAb titer of 64 MU/l (calibrated according to Medical Research Council research standard A 65/93) corresponding to an antigen binding capacity of 53 nmol Tg/MU (*Salabé et al* 1974) *FN* serum was diluted in a TgAb free human serum which was diluted 1/5 with the buffer used for the CC test (*Nielsen & Sie*

TABLE 1 *Age Distribution of 71 Subgrouped Patients with Thyroid Diseases and 63 Sex and Age matched Control Subjects*

Subgroup	Patients			Controls	
	Number		Age (years)	Number	Age (years)
	males	*females*	*Median (range)*		*Median (range)*
Nontoxic diffuse	0	23	*33*[a] (21–59)	22	*35.5* (23–69)
Nontoxic nodular	3	16	*52*[a] (28–66)	18	*51.5* (24–61)
Toxic diffuse	5	15	*53.5*[a] (18–74)	17	*51* (22–63)
Toxic adenoma	1	8	*63*[a] (28–75)	6	*46.5* (29–63)

a) No difference between the age of the patient groups and their appropriate controls (P > 0.1) Mann Whitney s rank sum test

antibody against Tg and IgG while their report contains no information as to the complement fixing or activating properties of the IC. We have shown the presence of both Tg and TgAb in serum following subtotal thyroidectomy of a patient with Graves disease (*Feldt Rasmussen et al.* 1979 b). The same serum specimens did not show any activity in the CC test. This might be due to insensitivity of the assay used (lower detection limit for preformed BSA anti BSA complexes in serum milieu is about 5 µg/ml). However, when *in vitro* preformed complexes composed of native 19S Tg (*Feldt Rasmussen* 1978) and TgAb were tested at high concentration no CC activity was demonstrable which indicated that these TgAb's were not complement fixing as shown by *Roitt et al.* (1978).

An indirect evidence for the possible antigenic composition of the complexes and their pathogenic potential (e.g. complement activation) could be obtained if a correlation between CC activity and a specific antigen or antibody was observed as shown in varicella zoster infected patients (*Nielsen et al.* 1979). In the present study we could not show any correlation between anticomplementary activity and serum concentrations of Tg or TgAb for the whole material. The number of CC positive patients in the subgroups were too small to evaluate. Further, the TgAb method used did not measure complex bound TgAb (*Feldt Rasmussen et al.* 1979) which limits the conclusions that can be made. The method for measurement of Tg also had a bias when testing sera containing TgAb even in low levels (*Schneider & Pervos* 1978, *Feldt Rasmussen et al.* 1979).

However, as suggested by our *in vitro* experiments, the CC activity demonstrated in the patient sera was probably not due to complexes with native Tg. This would be in agreement with the data of *Hopf et al.* (1978) who were unable to demonstrate Tg in complexes isolated by a Raji cell assay from patients with Graves disease during treatment. It would also be compatible with the data of *Calder et al.* (1974) demonstrating that the active factor responsible for the CC activity had a lower molecular weight than Tg. Whether this was a non Tg antigen or a fragment of Tg present in serum at least under some conditions (*Schneider & Frohman* 1976, *Feldt Rasmussen et al.* 1978) is at present unknown.

Interestingly, however, we could demonstrate a significant correlation in patients with Graves disease between CC activity and TSAb levels which may be involved in the pathogenesis of this disease (*Volpé* 1978). It has been [illegible]

a[illegible]

th[illegible]

fi[illegible]

TSH and TSAb activate the adenylate cyclase system through different pathways. Thus, the findings seem conflicting and the antigen is at present unknown.

The technical assistance of Lise Schrøder, Hanne Jensen, Jette Thinggård, Anni Hansen, Else Marie Skjold, Susanne Christensen and Jette Bark is gratefully acknowledged. The staff at the Blood bank of Odense University Hospital is thanked for collection of sera from control subjects.

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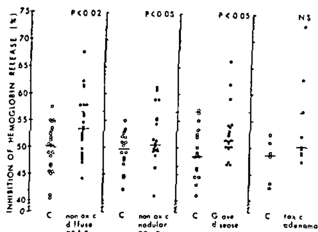


Fig 2 CC activity (per cent inhibition of hemoglobin release) in patients with nontoxic diffuse (n = 23) and nontoxic nodular goiter (n = 19) Graves' disease (n = 20) toxic adenomas (n = 9) and sex and age – matched control subjects The Mann Whitney's U test for independent samples was used
Dashed line indicates mean per cent inhibition of hemoglobin release + 2 SD of controls

distribution seemed to be composed of two populations, one of which (the group with the lower values) had a slope identical to the control group

After subgrouping of the patients the greatest difference between median values of controls and patients, was observed in the groups with nontoxic and toxic diffuse (Graves' disease) goiters confirming results from other investigators (*Brohee et al* 1978) However, unlike these we could not show any differences between disease groups, partially due to too small numbers of patients in each group

Recently, increased *in vitro* delayed type hypersensitivity in response to *Yersinia enterocolitica* serotype 3 has been demonstrated in Graves disease and in nontoxic diffuse goiter but not in nodular goiter (*Bech et al* 1977, 1978) These findings combined with the present results may suggest immunological similar reactions in the two groups of patients with diffuse enlargements of the thyroid as opposed to nodular, and irrespective of the hormonal activity

Calder et al (1974) tested sera from patients with thyrotoxicosis, Hashimoto's thyroiditis and primary hypothyroidism and found increased frequency of CC-activity in all groups compared with a sex and age matched control group The increased CC-activity in Graves' disease found in the present study confirms this finding Recently, *Hopf et al* (1978), by use of a Clq binding assay, reported that *practically all patients with Graves disease* had IC in serum during treatment It is, however, difficult to compare their results with the present findings, as the assays used differ, and *Hopf et al* only investigated two patients before treatment

Using a specific assay for detection of Tg IC *Takeda & Kriss* (1977) reported a higher frequency of IC positive reactions for patients with Graves'disease It is not known whether Tg IC were responsible for the CC-activity demonstrated by *Calder et al* (1974) The former authors identified the antigen and antibody of the complexes by using

TABLE 3 *Levels of Thyroglobulin (Tg) Thyroglobulin Antibodies (TgAb) and Thyroid Stimulating Antibodies (TSAb) in Serum from 71 Patients with Thyroid Diseases as well as Sex- and Age matched Control Subjects Medians and Ranges are Given*

	TgAb – positive		Serum Tg		Serum TSAb	
	Number of persons	Level (MU/l)	Number of persons[a]	Level (μg/l)	Number of persons	Level (%)
Nontoxic diffuse goiter (n = 23)	2	0 04 (0 02–0 05)	23	16 5 (2 7–83 2)	23	100 (60 2–112)
Nontoxic nodular goiter (n = 19)	4	0 04 (0 03–2 87)	18	20 1 (<1 7–99 0)	19	93 0 (68 2–129)
Toxic adenoma (n = 9)	1	0 04	9	33 9 (12 0–102)	9	104 (85 8–124)
Graves' disease (n = 20)	8	0 25 (0 03–0 63)	14	44 7 (11 7–55 0)	20	122 (91 0–198)
Controls (n = 63)	5	0 05 (0 01–0 23)	58	6 5 (<1 7–21 9)	63	94 3 (73 1–110)

a) Because of the presence of TgAb Tg was not measured in all sera

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DONOR-SPECIFIC CELL-MEDIATED CYTOTOXICITY IN RENAL ALLOGRAFT RECIPIENTS

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Dickmeiss E Donor specific cell mediated cytotoxicity in renal allograft recipients Acta path microbiol scand Sect C 87 371–376 1979

The immunological *in vitro* responses of cadaver kidney transplanted patients against donor specific target cells were investigated with the test systems for antibody-dependent cell mediated cytotoxicity (ADCC) and direct cell mediated cytotoxicity (DCMC) The test systems were used in 58 recipients just before the transplantation Nine recipients (17%) had positive donor directed ADCC reactivity and of these seven had early rejection episodes but this outcome was not significantly different from the outcome in the ADCC negative group Only one recipient showed positive donor directed DCMC reactivity and had early loss of the graft in rejection The test systems were further used to monitor the donor specific responces after the transplantation in 32 recipients Positive DCMC reactivities were significantly correlated with clinical rejections but most often positive reactions occurred too late to be of predictive value The donor specific ADCC reactivity after transplantation was not clearly correlated with rejections It is concluded that the predictive and diagnostic values of the two test systems were too weak to justify their use as routine methods in the clinical management of the kidney transplanted patient

Key words Cell mediated cytotoxicity kidney transplantation

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The *in vitro* test for direct cell mediated cytotoxicity (DCMC) and antibody-dependent cell mediated cytotoxicity (ADCC) have made it possible to test for donor specificic cell mediated cytotoxicity in human renal allotransplantation DCMC is a test for alloreactive cytotoxic T lymphocytes (CTL) (22) and ADCC is essentially a very sensitive test for alloantibody (2) Histopathology of rejected kidneys indicates that both cellular and humoral immune mechanisms plays a decisive role in rejection (16) It has been proposed therefore that the DCMC and ADCC tests might predict changes in graft function before irreversible deterioration occurs (9) and that they might elucidate the particular pathogenic mechan sm [illegible] these tests and to investigate whether *in vitro* immune responsiveness accurately reflects rejection activity in the renal transplant recipients

MATERIALS AND METHODS

Patients 58 cadaver kidney recipients treated at the State [illegible]

[illegible] negative The diagnosis of rejection was established by a significant reduction in the 24 h endogenous creatinine clearance in two consecutive determinations and in patients with anuria by exploratory surgery and biopsy In both groups every attempt was made to exclude other

sex and age measured by a modified radioimmunoassay Acta endocr (Kbh) *90* 440–450 1979a

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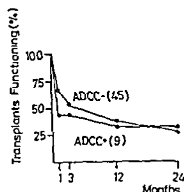


Fig 1 Long term prognosis of the patients tested with the ADCC cross match before transplantation.

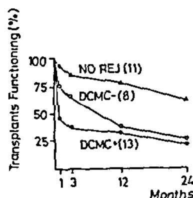


Fig 2 Long term prognosis of the patients monitored after the transplantation with the DCMC test No rejections DCMC negative throughout the course (▲–▲) Rejections diagnosed clinically but the patients were DCMC negative throughout the course (O–O) Rejections accompanied by positive DCMC tests (●–●)

Tests after transplantation A total of 32 patients were tested for donor specific DCMC twice weekly during the first postoperative month Twentyone of the patients had rejection episodes during this period and thirteen of these (approx 60%) had positive DCMC tests in relation to these episodes The eleven patients without rejection episodes never had positive DCMC's The correlation between positive DCMC and rejection thus is highly significant (Table 2) However, only five of the patients had their first positive DCMC test one or at most two days *before* rejection was clinically diagnosed The clinical course of the patients with rejection episodes accompanied by positive DCMC was not different from that of patients with rejection episodes without DCMC reactivity (Table 3 and Fig 2) except for a slightly but not significantly ($p = 0.2$) greater tendency in the former group to irreversible rejections In the group of patients with positive DCMC this reactivity became negative after about a week in patients with reversible rejections Of the seven patients with irreversible rejections five had

TABLE 2 *DCMC Tests and Rejections*

	Rejection	No rejection
Pos DCMC	13	0
Neg DCMC	8	11

her s exact test. $p = 0.0006$

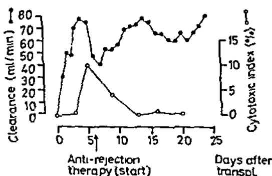


Fig 3 Results of DCMC tests in a case of reversible rejection

TABLE 3 *DCMC positive Rejections Compared with DCMC negative Rejections*

	The day posttransplantation rejection was diagnosed		Rejection was	
	median	range	irreversible	reversible
Pos DCMC	7	4–19 (N = 13)	7	6
Neg DCMC	6	4–10 (N = 8)	2	6

causes of reduction in kidney function by examination with isotope renography, intravenous urography, pyelography or angiography, or a combination of these The diagnosis of rejection was made indepently of the results of DCMC and ADCC tests All patients were treated with a standard immunosuppressive regimen consisting of azathioprine and corticosteroids Rejection crises were treated with 0 5–1 0 g methylprednisolone per day for 2–3 days, followed by prednisone, 300 mg/day, with gradual reduction depending on the response

Target cells The target cells in the tests performed before transplantation were lymphocytes separated by the ficoll hypaque method from a donor blood sample taken together with the sample used for tissue typing Target cells were labelled with ^{51}chromium by incubating approximately 1×10^6 lymphocytes with 200 μCi $Na_2{}^{51}CrO_4$ (Kieller, Norway Spec act 100–300 μCi/ μg) in a volume of 0 5 ml RPMI 1640 supplemented with 10% heat inactivated pooled human serum (RPMI-HS) for 45 minutes at 37 °C After labelling the cells were washed thrice and resuspended at the desired concentration in RPMI-HS In tests after transplantation target cells were from the donor spleen The spleen was removed after nefrectomy, cut in small pieces and stored in RPMI in a 1 l bottle at 4 °C over night During this incubation the spleen cells migrated out in the medium, and the lymphocytes were isolated from the medium by the ficoll hypaque method These lymphocytes were blast-transformed by incubating the cells in RPMI-HS (1×10^6 cells pr ml) for three days with PHA-P (Difco) at a concentration of 1 500 v/v The blast cells were frozen at a controlled rate and stored in liquid nitrogen in aliquots of 3×10^6 blast cells in ½ ml RPMI-HS with 10% DMSO as the cryoprotective agent Labelled target cells were prepared from thawed and thrice washed cryopreserved cells as above

DMCM Effector lymphocytes were isolated from heparinized recipient blood by the ficoll hypaque method The cells were washed thrice and resuspended in RPMI-HS In tests before the transplantation 2×10^6 effector cells and 5×10^4 target cells were incubated in a total volume of 1 ml RPMI-HS in round bottomed tubes for five hours at 37 °C in a moist atmosphere with 5% CO_2 In tests after transplantation a mixture of 1×10^6 effector cells and 1×10^4 target cells in a total volume of 300 μl were used After incubation the test tubes were centrifuged and supernatant and cell pellets counted separately in an auto-gamma counter The ^{51}chromium release were expressed as the amount of ^{51}Cr released in percentage of the amount originally incorporated in the target cells at the beginning of the experiment As spontaneous release, the percentages of ^{51}Cr released from cells incubated in medium alone were used All tests were done in triplicate

ADCC The ADCC-tests were done in parallel with the DCMC tests and used the same effector and target cells, but the incubation mixture contained in addition heat inactivated recipient serum in a final concentration of 20% Furthermore, as positive controls the target cells used were precoated with a multispecific human alloantiserum as previously described (4) and incubated with the effector cells in the same final cell concentrations The positive control was only performed in tests before transplantation

Calculation of results In the DCMC test the cytotoxic index (CI) was defined as per cent ^{51}Cr released from target cells in mixtures with effector cells minus per cent ^{51}Cr release in medium alone In the ADCC test the CI was per cent release in mixtures with effector cells and recipient serum minus per cent release in mixtures with effector cells alone Tests were regarded as positive if the CI was above 5%

RESULTS

Tests before transplantation A total of 58 recipients were tested immediately before transplantation for ADCC and DCMC activity against freshly explanted donor lymphocytes as targets In four cases the positive control tests (see Materials and methods) had release values below 10% and were discarded Positive control values of the remaining 54 patients were approximately 30% (median) with a range of 15% to 61% This indicates that the effector cell activity in ADCC (K-cell activity) of these recipients was of sufficient magnitude and that the donor target cells used were reliable The results of the donor specific ADCC-tests in these patients are compared with clinical outcome in Table 1 Nine (17%) of the 54 cases showed donor-specific ADCC activity There is a greater tendency for early acute rejection episodes in the group with positive ADCC, but this difference does not reach statistical significance The ADCC activities in the positive group ranged from 7% to 41% but there was no clear difference in the activities of those who rejected compared to those who did not reject the kidney There is no difference in the long term prognosis of the patients (Fig 1)

Only one of the recipients had a positive donor directed DCMC test (but no ADCC activity in her serum) and she experienced an early acute irreversible rejection

TABLE 1 *ADCC Cross Match Tests and Early Rejections*

	Rejections		No rejections
	Reversible	Irreversible	
Pos ADCC	1	6	2
Neg ADCC	9	12	24

Fischer's exact test (columns I and II grouped together) $p = 0.09$

the peripheral blood which are at the lower limit for detection in the DCMC test. This could be caused by (1) the immunosuppressive therapy (2) the possibility that the blood lymphocytes tested are depleted of CTLs which might be located in the graft or (3) the pathogenic CTLs might in som cases have specificity for determinants on the kidney which are not present on PHA blast (4) finally the rejection might be caused by other effector mechanisms than those determined by CTL. Unfortunately we are not able in the individual case to distinguish between these possibilities Since the DCMC positive acute rejections were not clinically different from the DCMC negative rejections the present investigation does not indicate that the test might be able to distinguish between pathogenetically diffe nt rejections

When the ADCC test was used as a monitoring

xplanations First the antibodies detected by this st might not always be detrimental to the graft as iscussed previously Second the choice of effector ells to use in the test is difficult Effector cell ctivity (K-cells) is reduced in immunosuppressed ecipients (5) and the use of the recipients own cells s effectors might therefore cause false negative esults On the other hand the use of third part ffector cells might also cause false negative reactions if the antibody in question by chance also is directed against the effector cell donor (4) Third the alloantibodies which are generated in immunosuppressed recipients may be of the IgM class (18) and therefore unable to activate the ADCC reaction or they might be directed against kidney specific antigens Fourth the rejection might be caused by other mechanisms than those determined by antibodies

In conclusion the predictive and diagnostic values of the two tests were too weak to justify their use as *routine* methods in the treatment of the transplanted patient and at the present time they should mainly be considered as tools in the research aimed at elucidating the immunological relationship between the recipient and his transplant

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The critical review of the manuscript by Dr *A Svejgaard* is gratefully acknowledged This work has been supported by grant no 512 8732 from the *Danish Medical Research Council*

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Fig 4 Results of DCMC tests in a case of irreversible rejection

continuous positive DCMC's until after the graftectomy Figs 3 and 4 *illustrate these points*

Eleven patients were also monitored for donor directed ADCC activity Five of the patients had no rejections, but two *of these had several positive* tests Of the six patients with *rejections, only four* had positive ADCC *tests in conjunction with rejection,* but five had *positive DCMC reaction* One developed ADCC reactivity in a quiet phase after the rejection was reversed and one developed ADCC reactivity after graftectomy Thus ADCC activity was not strongly correlated with active rejection

DISCUSSION

The ADCC and the DCMC tests have been used as pretransplant cross match tests and as monitoring *tests after transplantation in the present investigation*

The importance of pretransplant cross match tests has been *well established after it was shown* that complement dependent donorspecific antibodies in recipient serum at transplantation usually resulted in *hyperacute rejections* (13) However, there is evidence to suggest that some early acute rejections are connected with the occurrence of donor specific antibodies which are too weak to be detected in the complement dependent cytotoxicity (CDC) test (14 19) Furthermore, cellular presensitization is not necessarily reflected by the humoral state of presensitization Thus supplementary cross match tests are clearly warranted In accordance *with the fact that* ADCC is a very sensitive test for *alloantibodies* (4) several investigators have found that an appreciable number of recipients (10–20 %) *with a negative* CDC cross match in fact have a *positive ADCC cross match* However, there are differences in opinion as to the predictive value of an isolated positive ADCC *cross match Thus some* investigators have found a *significant* correlation between positive ADCC cross match tests and early transplant failures (11, 20, 21), *whereas other* did not find a correlation (12, 15) In the present investigation seven out of nine with an isolated positive ADCC *cross match had early acute* rejections but this outcome was not significantly different from the outcome in the ADCC negative group The ADCC *technique may in some cases* detect antibody with specificity for antigens exclusively presented by donor B lymphocytes and such *antibodies do not seem to cause early acute* rejections (6) Furthermore, it is not known if the *in vivo* counterpart of the ADCC reaction on its own is sufficient to cause *clinically evident rejections*, and the relationship between a positive *in vitro* activity and a subsequent rejection might very well be of an *indirect nature In the present* investigation four *recipients* with positive ADCC cross matches could be monitored after transplantation, and in fact three had positive DCMC tests in relation to their rejections On the other hand, it is clear that many early acute rejections are not preceded by a positive ADCC *cross* match Al together, this makes interpretation of any single ADCC cross match test less certain

A positive DCMC cross match has been correlated with poor graft outcome (8, 15) This was also seen in the present investigation However, it has been shown in animal experiments (1) and also in man (3), that direct cytotoxic T-lymphocytes (CTL) can *only be detected for a short period after* alloimmunization, although memory-CTL s circulates for a long time (1) Thus it is to be expected, that *the DCMC test is a less appropriate test for cellular* presensitization unless the recipient recently has received a blood transfusion

During the postoperative period there was a clear correlation between DCMC activity and rejection This has been seen in several other studies (7, 10 17) and thus indicates that CTL s plays a pathogenic role in many early rejections However as in the majority of the other studies, the posttransplant monitoring by DCMC had two shortcomings in the present investigation First DCMC reactivity was a late event most often *detected either at the same* time or shortly after the rejection was clinically evident Second, several clearcut rejections were *seen in spite of negative* DCMC reactions throughout the course The strength of the DCMC reaction when it was present was quite low (CI s of 6–20 %) compared with the reactivity which can be seen when normal individuals are immunized (3) *Together this indicates, that overt acute rejections* normally proceeds with concentrations of CTL's in

the peripheral blood which are at the lower limit for detection in the DCMC test This could be caused by (1) the immunosuppressive therapy, (2) the possibility that the blood lymphocytes tested are depleted of CTLs which might be located in the graft or (3) the pathogenic CTLs might in som cases have specificity for determinants on the kidney which are not present on PHA blast (4) finally the rejection might be caused by other effector mechanisms than those determined by CTL. Unfortunately we are not able in the individual case to distinguish between these possibilities Since the DCMC positive acute rejections were not clinically different from the DCMC negative rejections the present investigation does not indicate that the test might be able to distinguish between pathogenetically different rejections

When the ADCC [illegible]

explanations First the antibodies detected by this test might not always be detrimental to the graft as discussed previously Second the choice of effector cells to use in the test is difficult Effector cell activity (K-cells) is reduced in immunosuppressed recipients (5) and the use of the recipients own cells as effectors might therefore cause false negative results On the other hand the use of third part effector cells might also cause false negative reactions if the antibody in question by chance also is directed against the effector cell donor (4) Third the alloantibodies which are generated in immunosuppressed recipients may be of the IgM class (18) and therefore unable to activate the ADCC reaction or they might be directed against kidney-specific antigens Fourth the rejection might be caused by other mechanisms than those determined by antibodies

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INCREASED SERUM IgE IN HODGKIN'S DISEASE IS OF POLYCLONAL ORIGIN

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Landaas T Ø Grimmer Ø Heier H E & Godal T Increased serum IgE in Hodgkin s disease is of polyclonal origin Acta path microbiol scand Sect C 87 377–380 1979

The light chain type of serum IgE from 4 untreated patients with Hodgkin s disease with elevated IgE levels was studied by an immunoadsorbent technique Serum IgE was found to contain both kappa and lambda l ght chains in all cases studied In addition an association between serum levels of IgE and that of IgA IgG and IgM was demonstrated These findings make it unlikely that increased serum IgE in Hodgkin s disease is of monoclonal origin and support the view that serum IgE in such patients reflects a general d sturbance in the regulation of their humoral immune response

Key words Hodgkin s disease serum IgE

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Rece ved 23 v 79 Accepted 16 vii 79

The pathogenesis of Hodgkin s disease remains poorly understood Although Hodgkin and Reed Sternberg cells are regularly found they are usually extremely scarce while lymphocytes and often inflammatory cells predominate Moreover the origin of the Hodgkin cell itself is not clear Such cells have been found to contain polyclonal IgG i e the IgG contains both κ – and λ light chains

[illegible]

...odgkin cells may be of macrophage origin However since these cells do neither resemble macrophages morphologically (2 5) nor cytochemically (3 6 19) their derivation at this point remains unsettled

Among the unexplained immunological aberrations found in Hodgkin s disease are high levels of serum IgE of some patients (1 13 15 20 21) [illegible]

increased IgE in Hodgkin s disease is due to an expanded possibly neoplastically altered clone of IgE producing B cells we have studied the light chain composition of serum IgE in these patients by immunoadsorbent techniques

PATIENTS AND METHODS

Patients

Blood from 66 untreated patients with Hodgkin s disease was obtained from the Norwegian Radium Hospital Sera from four of the patients with elevated serum IgE were tested further by the immunoadsorbent technique Upon direct questioning these patients denied previous atopic symptoms In addition they had no reaction to lymphangiography There were no clinical or anamnestic evidence that any of these patients were suffering from parasitic infections Neither did the examination of their sera using a battery of 8 allergens (RAST) give any evidence for atopy

Normal Sera

Sera from 35 healthy blood donors were used as normal controls

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TABLE 1 *Total Serum IgE and IgE in Eluates from Anti κ and Anti λ and Control Immunoadsorbent Columns (U/ml)*[b]

Patient no	Total serum IgE[a]	Sepharose anti κ = IgE/λ	Sepharose anti λ = IgE/κ	IgE/λ IgE/κ	Control – IgE/λ + IgE/κ
P 133	1932	579 (480– 678)	1037 (983–1091)	1 7 3	1467 (1431–1503)
T 11	2338	552 (519– 585)	483 (430– 536)	3 4 3	1279 (1242–1316)
C 33	4314	1471 (1334–1608)	2262 (2043–2481)	2 3	3844 (3836–3852)
T 39	5063	1262 (1169–1355)	1610 (1532–1688)	2 4 3	3283

[a] Normal range Serum IgE < 100 U/ml
[b] Duplicate IgE values determined by RIST

highest value in the normal control group Sera from 4 of these were examined by immunoadsorbent columns The values for IgE/κ and IgE/λ in the eluates are summarized in Table 1 which also shows the total amounts of IgE in these sera

Table 1 shows that the total amounts of IgE passed through the anti light chain columns were about the same as in the eluate from the control column The IgE/λ IgE/κ ratio showed some variation from one serum to another (IgE/κ IgE/λ 1 7 3 to 3 4 3) but all sera appeared to contain both IgE/κ and IgE/λ within the range found for polyclonal serum IgG (16)

The amounts of IgG IgA and IgM were estimated in Hodgkin sera with low IgE (< 10 U/ml) and high IgE (> 400 U/ml) values The results are shown in Table 2 The group with high levels of IgE also had significantly higher concentration of IgG IgM and IgA However except IgG the concentrations of these latter immunoglobulins were still within the normal range

DISCUSSION

The nature and origin of the neoplastic process in Hodgkin s disease still remain obscure The process appears to be intimately linked with the Hodgkin and Reed Sternberg cells but the origin of these cells remains uncertain Such cells have been shown by a number of investigators to contain immunoglobulin (4 9 18) suggesting that they could originate from B lymphocytes However several authors (7 8 11 19) have reported that the IgG in these cells is not monoclonal and thus it is unclear whether the

TABLE 2 *Levels of Serum Immunoglobulins in Sera from Patients with Untreated Hodgkin s Disease Classified into Those with IgE < 10 U/ml and IgE > 400 U/ml*

Serum Group	Number	IgG (mg/100 ml) Mean ± s d Range	IgA (mg/100 ml) Mean ± s d Range	IgM (mg/100 ml) Mean ± s d Range
IgE < 10 U/ml	5[a]	1456 ± 128 1342–1617	216 ± 56 170–287	87 ± 16 62–103
IgE > 400 U/ml	7[b]	1843 ± 282 1595–2200	350 ± 106 222–505	167 ± 66 93–270
Student s t test		p < 0 01	p < 0 025	p < 0 0125

[a] Age 26–63 years (5 with nodular schlerosis and 2 with mixed cellularity
1 in stage I 3 in stage III and 1 in stage IV)
[b] Age 35–54 years (3 with nodular sclerosis 1 with lymphocyte predominance
and 1 with lymphocyte depletion 2 in stage II
3 in stage III and 2 in stage IV)

Normal values		
IgG		800–1800 mg/100 ml
IgA		90– 450 mg/100 ml
IgM	M	60– 250 mg/100 ml
	F	70– 280 mg/100 ml

Immunological Reagents

Rabbit anti human κ light chain (code no 10 9K2) rabbit anti human λ light chain (code no 10 9L2) and the immunoglobulin fraction from a non immunized rabbit (normal rabbit immunoglobulin code no X901) were obtained from Dakopatts (Copenhagen)

Immunoadsorbent Columns

Rabbit anti human κ light chain λ light chain and normal rabbit immunoglobulin the last one as a negative control were insolubilized by coupling to CNBr activated Sepharose 4B (Pharmacia Fine Chemicals Uppsala Sweden) The detailed procedure for coupling protein to CNBr activated Sepharose 4B is decribed in the booklet »Affinity Chromatography‹ (Pharmacia Fine Chemicals Uppsala (12)) Immunoglobulins with either κ or λ type of light chains were adsorbed from serum as follows Parallel samples of 50 µl serum from each patient were eluated through columns with either insolubilized anti κ anti λ or normal rabbit immunoglobulin by 0 05 M NH_4HCO_3 pH 8 0 The eluates from the anti light chain columns consisted of serum without immunoglobulins of the selected light chain type from the control column of unaltered serum These were consentrated to a fixed volume for IgE determination by freeze drying and solubilizing in the eluating buffer

Test of Column Capacity

The volumes of antibody reagent which were coupled to Sepharose particles were based on the antibody capacity (1 7 ml Dakotiter) With regard to the control the same protein amount as for anti light chains (20 mg) was used Preliminary experiments were performed to find out if this was sufficient to remove IgE/κ or IgE/λ respectively from 50 µl serum 50 µl serum from a normal control with a relatively high IgE content – 482 U/ml (RIST) determined after freeze drying and solubilization – was passed through an anti κ and anti λ column successively After passing through both columns the protein eluate was freeze dried and solubilized and the IgE content determined to be 10 U/ml (RIST) Therefore approximately all IgE was removed by passing through the anti κ and anti λ columns

IgE Determinations

Phadebas IgE PRIST ® was used in the determination of IgE concentration in untreated sera from patients with Hodgkin s disease and normal controls while RIST Phadebas® IgE Test was used in freeze dried and solubilized eluates and sera The anti IgE used was D_ϵ 2 specific and the analyses were performed according to the producer (Pharmacia AB Uppsala Sweden)

Specific IgE Antibody Tests

Sera were tested for IgE activity against timothy alder birch mugwort daisy mite cat and mold fungus (Cladosporium) by the Phadebas® Radioallergosorbent Test (RAST Pharmacia AB Uppsala Sweden) This was performed as described by the producer

Quantitation of Serum IgG IgM and IgA

The concentration of serum immunoglobulins was determined by the single radial immunodiffusion technique using Tri partigen plates (code no OTDS 03 OTOT 03 and OTDU 03 Behringwerke Marburg Germany) and standard solutions of IgG IgM and IgA (code no OTRA 07 OTRB 07 and OTRC 07 Behringwerke Marburg Germany) Each serum was assayed in duplicate

RESULTS

Fig 1 shows the IgE levels in sera from 66 patients with Hodgkin s disease and 35 normal controls Clearly 5 of the Hodgkin sera had markedly increased IgE values i e more than twice the

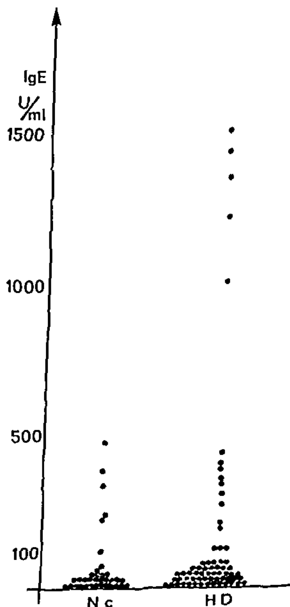


Fig 1 IgE levels in sera from untreated patients with Hodgkin s disease (H D) and norr
determined by PRIST

cta path microbiol scand Sect. C 87 381–389 1979

IN VITRO INFLUENCE OF ENDOTOXIN ON HUMAN MONONUCLEAR PHAGOCYTE STRUCTURE AND FUNCTION

I Depression of Protein Synthesis Phagocytosis of Candida albicans *and Induction of Cytostatic Activity*

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Hammerstrøm J & Unsgaard G *In vitro* influence of endotoxin on human mononuclear phagocyte structure and function I Depression of protein synthesis phagocytosis of *Candida albicans* and induction of cytostatic activity Acta path microbiol scand Sect C 87 381–389 1979

The effect of *E coli* endotoxin (LPS) on human monocytes and peritoneal macrophages (PEC) during *in vitro* differentiation was studied Short term (< 24 hours) exposure to LPS in concentrations up to 50 µg/ml did not affect monocyte survival or ^{51}Cr release but concentrations of 10 µg/ml or more reduced monocyte survival when LPS exposure was prolonged to 72 h Undifferentiated monocytes seemed to be more sensitive to this effect Monocyte and PEC protein synthesis was reduced by non toxic LPS concentrations in both undifferentiated and differentiated cells LPS exposure reduced monocyte ingestion and degradation of ^{125}I labelled *Candida albicans* dependent on time and dosage The induction of monocyte and PEC mediated cytostatic activity to tumour cells induced by prolonged *in vitro* culture was also impaired by LPS The morphological alterations induced in mononuclear phagocytes by LPS included a changed distribution of cells in the monolayer changes in membrane structure and apparent reduction of lysosomes LPS thus interferes adversely with several aspects of human mononuclear phagocyte *in vitro* differentiation

Key words Monocytes macrophages endotoxin cytostasis protein synthesis phagocytosis

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It has been reported that cell wall lipopolysaccharides from gram negative bacteria exert a variety of effects on immunological effector cells Focusing on mononuclear phagocytes two main bodies of seemingly contradictory evidence can be discerned Stimulating enhancing or »activating« *in vitro* effects of LPS on mononuclear phagocytes have been reported in terms of cell size and spreading (17) synthesis of lysosomal enzymes (7 17 23) content of cell protein (5) phagocytosis (2 17) pinocytosis (2 3) growth of mononuclear phagocyte cell lines (18) and cytotoxicity to tumour cells

(see 12 f ... [illegible]

20 24) The toxic effects have been reported in the same concentration range (µg/ml) as the stimulating effects and mononuclear phagocytes have even been regarded as especially prone to LPS toxicity (13) Moreover reports of LPS effects on macrophage function especially as regards macrophage mediated cytotoxicity to other cells often concentrate only on one aspect of macrophage function thereby leaving the apparent contradiction of LPS stimulation and toxicity unsolved In addition most

cells are derived from the B cell lineage or the macrophage cell lineage

Some patients with Hodgkin s disease appear to have exceptionally high levels of IgE in their serum (1, 13, 15, 20, 21) This immunoglobulin could be produced from a neoplastic clone of B lymphocytes Our investigation, however, clearly showed that the elevated IgE in sera from patients with Hodgkin s disease contained both κ and λ light chains Their IgE therefore apparently arises from more than one clone of IgE producing B cells

In some patients the high IgE concentration is associated with allergic manifestations (1, 21) but in the majority neither atopy (1) nor parasite infection (21) can explain their high levels of IgE

There have been several reports (13 20 21) suggesting that increased levels of IgE may reflect a defect in the regulatory T cell function in Hodgkin s disease This view is supported by the findings of others (15) which demonstrated a correlation between high serum IgE and impaired cell mediated immunity in patients with Hodgkin s disease

Our results show that the elevated serum IgE in these patients is of polyclonal origin Moreover an association was found between IgE levels and other immunoglobulin levels This further suggests that there may be a more general disturbance in the regulation of the humoral immune system at least in some patients with Hodgkin s disease

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IN VITRO INFLUENCE OF ENDOTOXIN ON HUMAN MONONUCLEAR PHAGOCYTE STRUCTURE AND FUNCTION

I Depression of Protein Synthesis Phagocytosis of Candida albicans *and Induction of Cytostatic Activity*

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The effect of *E coli* endotoxin (LPS) on human monocytes and peritoneal macrophages (PEC) during *in vitro* differentiation was studied Short term (< 24 hours) exposure to LPS in concentrations up to 50 µg/ml did not affect monocyte survival or ^{51}Cr release but concentrations of 10 µg/ml or more reduced monocyte survival when LPS exposure was prolonged to 72 h Undifferentiated monocytes seemed to be more sensitive to this effect Monocyte and PEC protein synthesis was reduced by non toxic LPS concentrations in both undifferentiated and differentiated cells LPS exposure reduced monocyte ingestion and degradation of ^{125}I labelled *Candida albicans* dependent on time and dosage The induction of monocyte and PEC mediated cytostatic activity to tumour cells induced by prolonged *in vitro* culture was also impaired by LPS The morphological alterations induced in mononuclear phagocytes by LPS included a changed distribution of cells in the monolayer changes in membrane structure and apparent reduction of lysosomes LPS thus interferes adversely with several aspects of human mononuclear phagocyte *in vitro* differentiation

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It has been reported that cell wall lipopolysaccharides from gram negative bacteria exert a variety of effects on immunological effector cells Focusing on mononuclear phagocytes two main bodies of seemingly contradictory evidence can be discerned Stimulating enhancing or »activating« *in vitro* effects of LPS on mononuclear phagocytes have been reported in terms of cell size and spreading (17) synthesis of lysosomal enzymes (7 17 23) content of cell protein (5) phagocytosis (2 17) pinocytosis (2 5) growth of mononuclear phagocyte cell lines (18) and cytotoxicity to tumour cells (see 12 for ref) On the other hand cytotoxic effe of LPS on mononuclear phagocytes were a pro nent feature especially in earlier reports (8 13 20 24) The toxic effects have been reported in same concentration range (µg/ml) as the stimu ting effects and mononuclear phagocytes have e been regarded as especially prone to LPS toxi (13) Moreover reports of LPS effects on mac phage function especially as regards macropha mediated cytotoxicity to other cells often conc trate only on one aspect of macrophage funct thereby leaving the apparent contradiction of l stimulation and toxicity unsolved In addition n

of the papers cited concern murine mononuclear phagocytes In this and the following paper (12) we have attempted to extend some of the observations made in animal systems to the human mononuclear phagocyte combining several assays in an attempt to correlate enhancing and adverse effects of LPS in the same completely human *in vitro* system

MATERIALS AND METHODS

Human monocytes were separated from venous blood of healthy adults by Ficoll/Isopaque centrifugation and plastic adherence as described in (9) The monocytes were cultured in 24 well tissue culture plates (Costar 3524 Costar Cambridge Mass USA well Ø 16 mm) ... d shes (Co ar 3506 Ø

ed human AB Rh + serum 0 1 mM l glutamine and 40 µg gentamicin per ml (HS M) The medium (0 5 ml in small wells *1 0 ml in dishes) was changed after one four* and eight days The monocyte purity of the monolayers was > 90% at culture start ~ 95% on day 4 and ~ 99% on day 8 (10) contaminating cells being almost exclusively lymphocytes after the first 24 hours of culture

Human peritoneal exudate cells (PEC) were separated from the sterile peritoneal exudate of a 60 year old woman in regular peritoneal dialysis The donor and the separation procedure have been described previously (9) The PEC (90% macrophages at culture start) were cultured in 24 well plates as described for monocytes

Endotoxin (LPS) treatment Lyophilized LPS from *E coli* serotype 026 B6 or 0128 B12 (Westphal phenol water extracted Sigma Chem Co St Louis USA) was ... 0 015 M NaCl and kept at −70 °C

medium was aspirated after shaking of the plate and LPS in 0 5 or 1 0 ml fresh HS M was added In the experiment shown in Fig 4A LPS diluted to six times the desired concentration was added in 0 1 ml HS M to the 0 5 ml already present in the well in order to avoid interfering effects of medium replacement *E coli* 026 B6 LPS was used unless otherwise stated

... phagocytes were

were photographed using a micro ... (25) in a phase contrast microscope (Leitz Laborlux) equipped with automatic photographic equipment (Leitz Orthomat) Some cultures were fixed in methanol and stained with May Grunwald Giemsa (MGG) before

with fresh HS M with 1 0 µCi ... Norway Sp act 1 0 Ci/mmol) The medium was withdrawn after a 4 hour incorporation period and the cells were harvested by hypotonic lysis and processed for liquid scintillation counting as described in (9)

Cell counts in monolayer culture were performed by counting plastic attached cells in an inverted phase contrast microscope (× 400) Ten random visual fields were counted per 35 mm dish To estimate viability 200 µl of 0 04% *trypan blue in 0 015 M NaCl* was added to monolayers in 16 mm wells and three visual fields in determined areas of the well were counted enumerating total cell count and percentage of cells taking up trypan blue As uptake of trypan blue increased with time counting was performed between 1 and 6 minutes after the addition of trypan blue In some experiments nuclei of adherent cells were counted in an automatic particle counter (Coulter Model Fn) by lysing the adherent cells with 0 1 M sodium citrate after removal of non adherent cells by two washes with HS M

Assay for LPS toxicity on monocytes Monocyte monolayers were labelled with ^{51}Cr (Kjeller Norway Sp act 166 67 mCi/mg) by incubating 4 day-old cultures with HS M containing 100 µCi ^{51}Cr/ml for 2 hours followed by two washes with Hanks balanced salt solution (HBSS) The monocytes were then incubated for 7 hours with or without LPS Supernatant and adherent cell radioactivity were determined separately in a Packard gamma radiation counter by lysing the adherent cells with 0 5% sodium dodecyl sulphate (SDS) after withdrawal of the supernatant The results are expressed as percentage release

$$\frac{\text{cpm supernatant}}{\text{Total releasable cpm}} \times 100$$

Total releasable cpm is the mean of (cpm supernatant + cpm SDS) of all wells in the experiment Spontaneous release was ~ 2 2% per hour

Phagocytosis and digestion of Candida albicans were assayed by a modification of the method described by *Viken & Ødegaard (22) As monocytes adhere* better to plastic surfaces than to glass (15) the assay was performed on monocytes in 16 mm wells without coverclips ^{125}I labelled *Candida albicans* was added to monocytes cultured for four days by replacing the medium with 0 3 ml of fresh HS M with 3 33 × 10^6 *Candida* particles per ml Ingestion was allowed for a 15 minute period and extracellular particles were then removed by three washes with HBSS One half ml of fresh HS M with or without LPS was added and the digestion of ingested particles was allowed to proceed for 24 hours The cultures were harvested by adding 0 5 ml of HBSS to all wells and then transferring the supernatants to test tubes which were centrifuged for 10 min at 2000 G The cells were harvested by hypotonic lysis for 10 min followed by vigorous pipetting Radioactivity was determined separately in the cell free *supernatant the sediment of* detached cells and the adherent cell lysate by gamma radiation counting

Total ingestion per culture was calculated as

(cpm adherent cells + cpm supernatant + cpm sediment)

Digestion capacity was calculated as percentage of [in]gested ^{125}I released to the supernatant during the [di]gestion period

$$\frac{\text{cpm supernatant}}{\text{cpm adherent cells + cpm supernatant + cpm sediment}}$$

Undigested intracellular *Candida* was calculated as [pe]rcentage of ingested ^{125}I found in the adherent cell [ly]sate

$$\frac{\text{cpm adherent cells}}{\text{cpm adherent cells + cpm supernatant + cpm sediment}}$$

Cell detachment was calculated as percentage of [in]gested ^{125}I found as undigested particles in dead [d]etached cells

$$\frac{\text{cpm sediment}}{\text{cpm adherent cells + cpm supernatant + cpm sediment}}$$

Total ingestion per culture was 22614 ± 3580 cpm (n = 5)

Assay for monocyte mediated cytostasis was performed as described previously (21) Briefly 10^5 cells of the human tumour-derived cell line NHIK 3025 in exponential growth were added to monocyte monolayers (35 mm dishes) [illegible]

[illegible] poration of radioactivity in target cells cultured with monocytes as percentage of incorporation in target cells cultured alone

$$\frac{\text{(cpm NHIK 3025 + monocytes)}}{\text{(cpm NHIK 3025)}} \times 100$$

Monocyte ^{3}H TdR incorporation was ignored in the calculations since this did not exceed 3% of the incorporation in target cell controls Target cell ^{3}H TdR incorporation in the control cultures was 173666 ± 12932 cpm (mean ± S E M of all experiments)

Statistics Results given are mean ± S E M of n experiments performed in triplicate (16 mm wells) or duplicate (35 mm dishes) P values were obtained by Wilcoxon s signed rank test for paired samples

RESULTS

Toxicity Studies on Human Monocytes

Relatively short term incubation (7 hours) in LPS in concentrations up to 50 µg/ml did not increase ^{51}Cr release from 4-day-old monocytes (Table 1) nor did it induce leakage of the cytoplasmic enzyme LDH (data not shown) Adherent cell counts after 24 hour exposure to the same concentration range did not decrease whether examined by visual counting of live cells or by automatic counting of adherent cell nuclei (Table 1) Trypan blue exclusion was not affected by 24 hour exposure of concentrations up to 50 µg/ml

Adherent cell counts after 72 hour exposure to LPS concentrations up to 1 µg/ml also remained

TABLE 1 *Lack of Influence of Short term Exposure to LPS on Monocyte Survival and ^{51}Cr release*

LPS concentration µg/ml	[a]Numbers of adherent cells/well × 10 ^{5}	[b]% release of ^{51}Cr from monocytes	[c]% of adherent cells taking up trypan blue
0	1 58 ± 0 10	17 0 ± 1 9	1 8 ± 1 0
0 01	1 57 ± 0 06	17 1 ± 3 5	0 9 ± 0 8
0 1	1 64 ± 0 02	18 5 ± 2 7	2 2 ± 1 0
1	1 52 ± 0 12	19 8 ± 3 1	1 0 ± 0 5
10	1 52 ± 0 13	17 3 ± 1 8	1 5 ± 0 8
50	1 52 ± 0 11	19 6 ± 1 2	1 6 ± 0 4

Monocytes were [illegible]
a Cultures [illegible] nuclei cou[illegible]
b Monocytes labelled with ^{51}Cr on day 3 of culture were exposed to LPS for 7 hours before harvesting n = 3
c Monocytes were incubated for 24 hours with LPS before assay n = 3
Mean ± S E M

TABLE 2 *Influence of Long-term Exposure to LPS on Monocyte Survival*

	LPS concentration µg/ml	LPS *preparation*	Number of monocytes per culture as % control without LPS	
			Day 4	Day 11
1	0 01	026 B6 (W)	103 0 ± 7 8	95 1 ± 2 2
	0 1	026 B6 (W)	105 2 ± 10 2	91 7 ± 3 1
	1	026 B6 (W)	95 2 ± 7 8	95 3 ± 5 7
2	50	026 B6 (W)	43 5 ± 5 9	57 0 ± 4 1
	50	0128 B8 (W)	37 4 ± 2 0	59 6 ± 10 8

Monocytes cultured with LPS for 72 hours before assay Mean ± S E M
1 Visual counting in 35 *mm* dishes *n* = 6
2 Automatic counting of adherent cell nuclei in 16 mm wells n = 2

unchanged (Table 2), but a reduction in the number of cells and an obvious detachment of monocytes were noted at 10 µg/ml and higher concentrations after this long-term exposure On account of the *reduced survival found at concentrations > 1 µg/*ml with long-term exposure, this concentration was chosen as the upper limit for the rest of the experiments

Effect of LPS on PEC and Monocyte Morphology

Incubation in LPS induced pronounced morphological changes in the mononuclear phagocyte cultures, beginning a few hours after the addition of LPS In monocyte cultures cytoplasmic membrane contraction was the most conspicuous feature, *many cells assuming spindle shape (Fig 1a and b)*

In the PEC cultures, the cells accumulated in clusters clearly visible with the naked eye (Fig 1c and d) The single cell showed contraction of the peripheral cytoplasmic veil which became greatly ruffled, with numerous thin needlelike membrane projections and blebs (Fig 1d and e) The number of perinuclear lysosomal granules seemed to diminish in LPS treated cells both in PEC (Fig 1d and e) and in monocyte cultures

Effect of LPS on Mononuclear Phagocyte Protein Synthesis

Preincubation with LPS reduced mononuclear phagocyte *protein synthesis* in a dose dependent manner, both in monocytes and PEC (Fig 2 and 3) The relative reduction was greatest in differentiated cells, where the protein synthesis is more intense

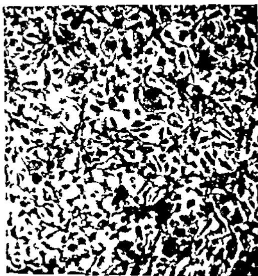
Effect of LPS on Monocyte Phagocytosis of [125]I-labelled Candida albicans

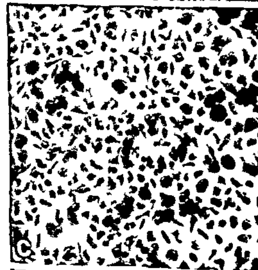
This effect was examined by three different protocols, since the time of LPS addition has been shown to be important for the effect produced (12 19) Preincubation with LPS before challenge with *Candida* reduced the number of particles ingested in a time dependent manner (Fig 4A) The digestion capacity *was also reduced, and the amount of* undigested *Candida* in adherent cells and the cell detachment after phagocytosis increased slightly (Fig 4A) These effects were also dose-dependent as can be seen in Fig 4B, where the cells were preincubated for a fixed time (24 hours) in varying doses of LPS To enhance monocyte mediated *cytostasis and cytolysis of target cells, LPS must be* present during the assay (12) LPS present during the digestion period did not produce any enhancement of the breakdown of ingested *Candida* (Fig 4C) On the contrary, a trend towards reduction of digestion capacity and increase in retained intracellular isotope was observed

Effect of LPS Preincubation on Monocyte-mediated Cytostasis

The cytostatic activity of human monocytes induced by *in vitro* culture in HS M (21) was reduced by 72-hour preincubation in LPS, the target cells escaping the cytostatic influence of the monocytes in a dose-dependent manner when the monocytes had been exposed to LPS before the assay (Fig 5) It should be noted that no LPS was *present during the monocyte/target cell interaction*

Fig 1 First row Control (a) or LPS [illegible] treated (d) PEC Live cells × 100 [illegible] incubated with LPS 1 µg/ml for 72 hours before photography with phase-contrast [illegible]



b

c

d

e

f

TABLE 2 *Influence of Long-term Exposure to LPS on Monocyte Survival*

	LPS concentration μg/ml	LPS preparation	Number of monocytes per culture as % control without LPS	
			Day 4	Day 11
1	0 01	026 B6 (W)	103 0 ± 7 8	95 1 ± 2 2
	0 1	026 B6 (W)	105 2 ± 10 2	91 7 ± 3 1
	1	026 B6 (W)	95 2 ± 7 8	95 3 ± 5 7
2	50	026 B6 (W)	43 5 ± 5 9	57 0 ± 4 1
	50	0128 B8 (W)	37 4 ± 2 0	59 6 ± 10 8

Monocytes cultured with LPS for 72 hours before assay Mean ± S E M
1 Visual counting in 35 mm dishes n = 6
2 Automatic counting of adherent cell nuclei in 16 mm wells n = 2

unchanged (Table 2), but a reduction in the number of cells and an obvious detachment of monocytes were noted at 10 μg/ml and higher concentrations after this long-term exposure On account of the reduced survival found at concentrations > 1 μg/ml with long-term exposure, this concentration was chosen as the upper limit for the rest of the experiments

Effect of LPS on PEC and Monocyte Morphology

Incubation in LPS induced pronounced morphological changes in the mononuclear phagocyte cultures, beginning a few hours after the addition of LPS In monocyte cultures, cytoplasmic membrane contraction was the most conspicuous feature, many cells assuming spindle-shape (Fig 1a and b)

In the PEC cultures, the cells accumulated in clusters clearly visible with the naked eye (Fig 1c and d) The single cell showed contraction of the peripheral cytoplasmic veil, which became greatly ruffled, with numerous thin needlelike membrane ... be (Fig 1d and e) The number of ... ish ... and

in monocyte cultures

Effect of LPS on Mononuclear Phagocyte Protein Synthesis

Preincubation with LPS reduced mononuclear phagocyte protein synthesis in a dose-dependent manner, both in monocytes and PEC (Fig 2 and 3) The relative reduction was greatest in differentiated cells, where the protein synthesis is more intense

Effect of LPS on Monocyte Phagocytosis of [125]I-labelled Candida albicans

This effect was examined by three different protocols, since the time of LPS addition has been shown to be important for the effect produced (12, 19) Preincubation with LPS before challenge with *Candida* reduced the number of particles ingested in a time-dependent manner (Fig 4A) The digestion capacity was also reduced, and the amount of undigested *Candida* in adherent cells and the cell detachment after phagocytosis increased slightly (Fig 4A) These effects were also dose-dependent, as can be seen in Fig 4B, where the cells were preincubated for a fixed time (24 hours) in varying doses of LPS To enhance monocyte-mediated cytostasis and cytolysis of target cells, LPS must be present during the assay (12) LPS present during the digestion period did not produce any enhancement of the breakdown of ingested *Candida* (Fig 4C) On the contrary, a trend towards reduction of digestion capacity and increase in retained intracellular isotope was observed

Effect of LPS Preincubation on Monocyte-mediated Cytostasis

The cytostatic activity of human monocytes induced by *in vitro* culture in HS-M (21) was reduced by 72-hour preincubation in LPS, the target cells escaping the cytostatic influence of the monocytes in a dose-dependent manner when the monocytes had been exposed to LPS before the assay (Fig 5) It should be noted that no LPS was present during the monocyte/target cell interaction

Fig 1 First row Control (a) or LPS ... (a) or LPS-treated (d) PEC Live cells × 100 ... incubated with LPS 1 μg/ml for 72 hours before photography with phase-contrast ...

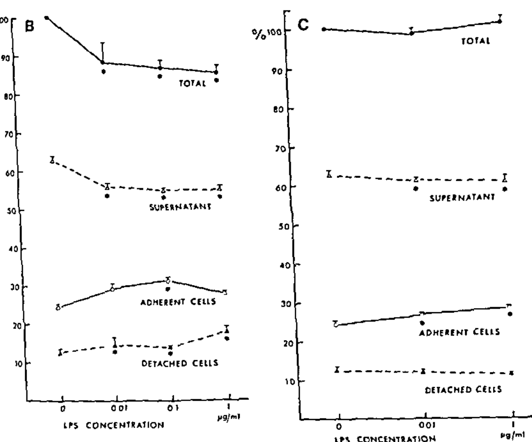


Fig 4 Influence of LPS on monocyte phagocytosis and digestion of ^{125}I labelled *Candida albicans* added on day 4 of monocyte culture Mean of triplicate cultures ●——● Total ingested ^{125}I as % of control without LPS △——△ Supernatant ^{125}I as % of total ingested ^{125}I (digestion capacity) O——O Cell associated ^{125}I in adherent cells after the digestion period as % of total ingested ^{125}I (undigested *Candida*) ▲——▲ Sediment ^{125}I as % of total ingested ^{125}I (undigested *Candida* in detached cells)
A Influence of preincubation with LPS 0 1 µg/ml for various times before addition of *Candida*
B Dose response curve of preincubation with LPS for 24 hours before addition of *Candida*
C Dose response curve of incubation with LPS during the digestion period LPS added immediately after ingestion of *Candida* on day 4 of monocyte culture
Points indicated by * differed significantly ($p < 0\ 05$) from control without LPS in five experiments

human AB serum Significant ($p \leq 0\ 031$) depression of protein synthesis cytostatic activity and *Candida* ingestion and digestion of a magnitude similar to that described with untreated serum was still produced by LPS preincubation (data not shown)

DISCUSSION

LPS toxicity to murine macrophages from LPS responsive strains has been reported at 5 µg/ml (8) and even lower (24) concentrations after 24 hour LPS exposure We were not able to demonstrate such short term toxic effects on human cells Experiments with one of the LPS preparations shown to be effective in the mouse (*E coli* 0128 B12 LPS (W)) (8) gave similar results However long term LPS exposure (72 hours) clearly reduced monocyte survival at concentrations higher than 1 µg/ ml undifferentiated cells being more sensitive to toxic LPS concentrations as reported by *Wiener & Levanon* (24)

The reduced protein synthesis found at non toxic concentrations contrasts with the findings of *Wiener & Levanon* (24) Moreover the reduced ability to degrade ingested *Candida albicans* after LPS exposure indicates that LPS reduces intracellular lysoso-

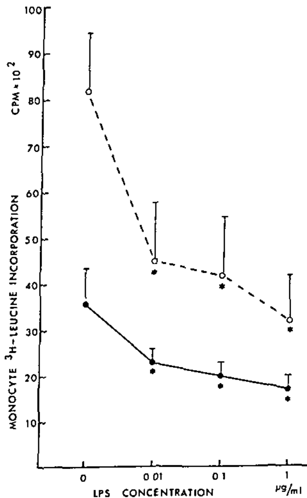


Fig 2 Influence of LPS on protein synthesis in monocytes assayed on day 4 (●——●) and day 11 (O--O) of monocyte culture Monocytes cultured with LPS for 72 hours before assay 35 mm dishes Mean ± S E M n = 6
*p < 0 05 as compared to control without LPS

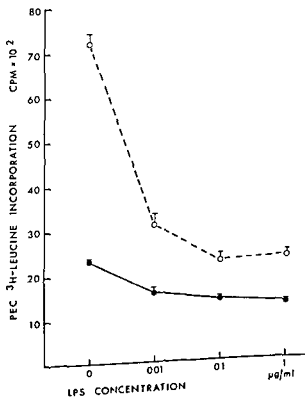


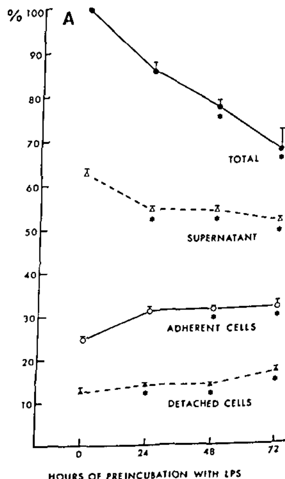
Fig 3 Influence of LPS on protein synthesis in human peritoneal macrophages cultured with LPS for 48 hours before assay on day 3 (●——●), or cultured with LPS for 72 hours before assay on day 8 (O---O) 16 mm dishes Mean ± S E M of triplicate cultures

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in these experiments Similar results were obtained in experiments with human PEC (data not shown)

Effect of Heat Inactivation of Serum

The effect of LPS on monocyte protein synthesis, *Candida* phagocytosis and induction of cytostatic activity by *in vitro* culture was tested in at least five separate experiments of each type, with *medium* supplemented with heat inactivated (56 °C, 30 min)



prolonged exposure to higher concentrations of LPS These observations like those in some of the cited papers (8 24) indicate that the concept of LPS as a general potentiator of all aspects of mononuclear phagocyte physiology is oversimplified

The technical assistance of *M Sorensen A Remen* and *B Lippe* is gratefully acknowledged We are indebted to Professor *J Lamvik* for discussion and help This work was supported by grants from the *Norwegian Research Council for Science and Humanities* the *Norwegian Cancer Society* and the *Norwegian Society for Fighting Cancer* J H is a research fellow of the *Norwegian Cancer Society* and G U a research fellow of the *Norwegian Research Council for Science and Humanities*

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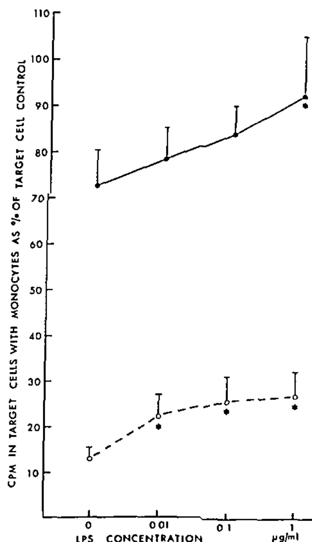


Fig 5 Influence of monocyte preincubation with LPS for 72 hours on the cytostatic influence on NHIK 3025 cells ^{3}H-TdR-incorporation in NHIK 3025 with 4 day-old (●——●) or 11-day old (O--O) monocytes is expressed as % of incorporation in *NHIK 3025 cultured alone Mean ± S E M n = 5*
* p < 0 05 as compared to control without LPS

mal enzyme activity in our system This contrasts with reports of increased lysosomal enzyme synthesis and activity in animal cells (17, 24) *Macrophage* sensitivity to LPS toxicity is genetically controlled in mice (8) *Whether* the observed discrepancy is due to genetically determined species variation in the response to LPS or to methodological differences cannot be ascertained at present The morphological observation of an apparent reduction in the number of lysosomal granules in *LPS-treated cultures* is in line with the data from the digestion assay

The *pronounced* alterations in cell distribution in the monolayer and individual cell morphology suggest that LPS influences mononuclear phagocyte membrane structure and function *The reduced* ingestion of *Candida* particles might also | explained as a membrane effect LPS has bee shown to bind to macrophage membranes an liposomes (3) *and to induce transmembrane* poter tial changes in human macrophages (6) There wa no short-term increase in macromolecular mem brane permeability as measured by ^{51}Cr-releas Selective release of enzymes in LPS-treated mono nuclear phagocytes has been described in anima (23) and human (7) cells, and this might be partl *responsible for the decline in lysosomal* granule and digestive ability found in our experiments

The reduced culture-induced cytostatic activity effected by LPS indicates that exposure to LPS interferes with most aspects of the human mono nuclear phagocyte differentiation induced by *in vitro* culture in our system (9, 21, 22, 25) We have demonstrated previously that such differentiation is not a prerequisite for lymphokine activation of cytostatic activity in monocytes (11), thus indicating that there may be at least two different pathways of induction and possibly also expression of human monocyte-mediated tumour cell inhibition LPS clearly interferes adversely with the induction of *differentiation-related* cytostasis *The observation* that LPS preincubation is unable to render human monocytes cytostatic disagrees with some earlier reports on animal cells (1, 4, 19) Preliminary experiments indicate that a very short preincubation period (2 hours) in LPS enhances monocyte cytostatic activity In our opinion, such rapid effects *would probably reflect the enhancement of cytosta-*tic and cytotoxic expression demonstrated in the accompanying paper (12) After completion of this work, *Horwitz et al* (14) reported induction of monocyte-mediated cytotoxicity by prolonged incubation in LPS-treated serum Differences in experimental design notably the use of heterologous serum, *make it difficult to compare these results* with ours In our hands, the use of serum-free media, or media supplemented with commercial heterologous sera or heat-inactivated human serum, seriously impair monocyte differentiation and fre*quently lead to increased cell loss when used for* prolonged culture (21), as also found by *Johnson et al* (15) The experiments performed with medium supplemented with heat-inactivated human serum indicate that the LPS effects are not dependent on LPS activation of heat-labile serum-derived complement components

The conclusion from the experiments presented here is that LPS influences human monocyte *in vitro* differentiation adversely in terms of cell growth, development of effector functions like particle ingestion and digestion and cytostatic influence on *tumour cells Cell* survival is also reduced by

Acta path microbiol scand Sect. C 87 391–399 1979

IN VITRO INFLUENCE OF ENDOTOXIN ON HUMAN MONONUCLEAR PHAGOCYTE STRUCTURE AND FUNCTION

2 Enhancement of the Expression of Cytostatic and Cytolytic Activity of Normal and Lymphokine-activated Monocytes

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Hammerstrøm J *In vitro* influence of endotoxin on human mononuclear phagocyte structure and function 2 Enhancement of the expression of cytostatic and cytolytic activity of normal and lymphokine activated monocytes Acta path microbiol scand Sect C 87 391–399 1979

The presence of non toxic concentrations of *E coli* endotoxin (LPS) during the *in vitro* interaction of normal human monocytes and a human tumour cell line (NHIK 3025) enhanced monocyte mediated target cell cytostasis and cytolysis Monocyte responsiveness to LPS was greatest at an intermediate stage of *in vitro* differentiation The expression of cytostatic and cytolytic activity by human monocytes activated with mediators from *Corynebacterium parvum* stimulated human lymphocytes was also enhanced by LPS Lymphokine activation did not induce additional LPS responsiveness in the monocytes Monocytes activated with lymphokines and subsequently deactivated by *in vitro* culture did not show any increase in LPS responsiveness A soluble cytostatic factor which is probably not cold thymidine was released from monocytes exposed first to lymphokines and then to LPS While LPS is ineffective as an induction signal of monocyte cytotoxicity to tumour cells in this system it enhances the expression of cytotoxicity induced by prolonged *in vitro* culture or lymphokine activation

Key words Monocytes human endotoxin lymphokines cytotoxicity

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Mononuclear phagocytes are recognized as a class of immunological effector cells that may be important in host control of tumour cell growth (5) A number of biological agents may stimulate mononuclear phagocytes *in vitro* to become nonspecifically cytostatic or cytolytic (13) for tumour target cells During the last few years several groups (10 17 19) have delineated a model for the *in vitro* activation of murine macrophages to tumouricidal effector cells Interaction with lymphokines (macrophage activating factor, MAF) is generally recognized as an important step in the induction of tumouricidal murine macrophages (2, 10, 17), while, according to some investigators, endotoxin (LPS) may act as a signal modulating the expression of tumouricidal activity (10, 17, 19) Prior exposure to lymphokines induces increased [illegible] induce tumouricidal murine macrophages by themselves (1 2, 21) We have shown previously that MAF can induce cytostatic ability in human monocytes (8 22) In the previous paper (9) we demonstrated that the cytostatic ability induced in human monocytes during their *in vitro* differentia

$$\% \text{SL} = \frac{\text{supernatant cpm (monocytes + NHIK 3025)} - \text{supernatant cpm (NHIK 3025)}}{\text{Total incorporated cpm}}$$

Total incorporated cpm was 14007 ± 3759 (n = 6) Controls of target cells with LPS were included in all experiments and % SL in monocyte/target cell cocultures with LPS was calculated using the spontaneous release in these controls LPS did not influence significantly the spontaneous release from target cells in the absence of monocytes (24 6 ± 1 2% of total incorporated cpm in target cells in HS M 23 8 ± 1 4% target cells with LPS 0 1 ug/ml assayed after 48 hours n = 6)

Statistics

All experiments were performed in triplicate Results are given as mean ± S E M of n experiments P values were obtained by Wilcoxon s signed rank test for paired samples

RESULTS

Effect of LPS on the Cytostatic Activity of Normal Monocytes

The impairment of monocyte mediated cytostatic activity produced by the presence of LPS during monocyte *in vitro* differentiation (9) was observed consistently with LPS pre incubation periods of 24–72 hours (Fig 1) If LPS was added to monocytes after 4 days of culture in HS M concomitantly with the target cells an enhancement of cytostatic activity was noted (Fig 1) Very short (2h) LPS preincubation before target cell addition also produced enhancement (data not shown)

LPS sensitivity of Monocytes at Different Stages of in vitro *Differentiation*

The enhancement observed with LPS present in the coculture of monocytes and target cells was found consistently and with monocytes at all stages of differentiation (Fig 2) Four-day-old monocytes at an intermediate stage of differentiation were most

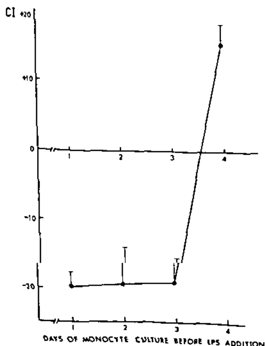


Fig 1 ... S E M n = 4

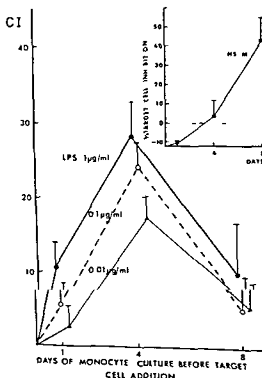


Fig 2 Cytostatic activity (CI) induced by addition of LPS simultaneous with target cells to monocytes cultured for various times ▲—▲ LPS 0 01 µg/ml O- - -O LPS 0 1 µg/ml ●—● LPS 1 µg/ml Percentage target cell inhibition of normal monocytes in the same experiments is shown in the insert Mean + S E M n = 6

tion to large macrophage-like cells (7, 23) is impaired by the presence of LPS during induction In this study, we report the results of experiments designed to examine the effect of LPS on the expression of human monocyte- and macrophage-mediated tumour cell cytostasis and cytolysis, and to investigate whether prior lymphokine activation modulates the LPS response in human cells

MATERIALS AND METHODS

Human Monocytes

The cells were separated from venous blood of healthy adults by Ficoll/Isopaque centrifugation and plastic adherence as described in (7) The monocytes were cultured in 24-well tissue culture plates (Costar 3524, Costar, Cambridge Mass, USA, well Ø 16 mm) as described in (8) in RPMI 1640 (Gibco, Bio Cult, Glasgow, Scotland) supplemented with 25% pooled human AB Rh + serum, 0 1 mM l-glutamine and 40 μg gentamicin per ml (HS-M) The medium (0 5 ml) was changed after one, four and eight days The percentage of monocytes in the monolayers was > 90% at start of culture, ~95% on day 4, and ~99% on day 8, contaminating cells being almost exclusively lymphocytes after the first 24 hours of culture

Human Lymphocytes

The non adherent cells aspirated after 90 min incubation of mononuclear blood cells on plastic (8) were adjusted to 10^6 cells/ml in HS-M Lymphokine supernatants were produced by culturing 4 ml of the cell suspension with killed *Corynebacterium parvum* strain

cate 1 ml cultures was assayed after lymphocyte culture

... had n (8) All lymphocyte donors

stimulated

n = 15)

Lymphokine (MAF) Activation of Monocytes

The medium was aspirated from 3 day-old monocyte cultures 0 25 ml HS-M followed by 0 25 ml filtered lymphokine supernatant were added, and the cultures were incubated for 24 hours

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Co, St Louis, USA)

described (9) The medium was aspirated from monocyte cultures and 0 25 ml of HS-M with or without target cells was added followed by 0 25 ml of LPS diluted to twice the desired concentration in HS-M In the experiments shown in Fig 1 and Fig 6, 0 1 ml of LPS diluted to six times the desired concentration was add[ed] to the 0 5 ml already present in the well, in order [to] avoid interfering effects of medium replacement

Assay for Monocyte-mediated Cytostasis

The human cell line NHIK 3025, originating from carcinoma in situ of the cervix (14) was used as targ[et] cells as described in (8) Briefly, 10^4 target cells in 0 2[5] ml HS M were added to monocyte cultures afte[r] aspiration of the medium, resulting in effector/target cel[l] ratios of approximately 10 1 Target cell DNA-synthesi[s] was determined by adding 1 μCi methyl-^{3}H thymidin[e] (methyl-^{3}H-TdR, sp act 5 Ci/mM, Radiochemica[l] Centre, Amersham, England) for the last five hours of [a] 24-hour coculture period of monocytes and target cells and processing the cultures for liquid scintillation counting as described in (7)

The results are expressed as cytostatic index (CI) which describes the cytostatic activity of LPS o[r] lymphokine-treated monocytes as compared to norma[l] monocytes cultured in HS M

$$CI = 100 - \frac{\text{cpm (treated monocytes + NHIK 3025)}}{\text{cpm (untreated monocytes + NHIK 3025)}} \times$$

The CI eliminates the inherent cytostatic activity of normal monocytes and focuses on the effects of treatment The cytostatic activity of normal monocytes is given separately for each series of experiments as percentage target cell inhibition

$$100 - \frac{\text{cpm (untreated monocytes + NHIK 3025)}}{\text{cpm (NHIK 3025)}} \times 100$$

Monocyte methyl ^{3}H-TdR incorporation was ignored in the calculations, since this did not exceed 5% of target cell incorporation Target cell controls with appropriate LPS concentrations were included in each experiment, but LPS did not influence target cell proliferation in the absence of monocytes (Target cell cpm in HS M 59697 ± 3964 cpm target cell cpm in HS-M with LPS 1 μg/ml 59712 ± 4138 cpm Mean ± S E M n = 36)

Assay for Monocyte-mediated Cytolysis

NHIK 3025 cells in exponential growth were labelled for 24 hours with 1 μCi/ml methyl ^{3}H-TdR Washed prelabelled target cells (10^4/well) with or without LPS 0 1 μg/ml were added to washed normal or lymphokine treated monocyte monolayers as described for the cytostasis assay Release of incorporated methyl ^{3}H-TdR was measured in the supernatant removed after 24 48 or 72 hours from cocultures of monocytes and target cells by liquid scintillation counting Spontaneous release was measured in the supernatant of 10^4 prelabelled target cells plated alone Total incorporated cpm was determined by lysing target cell monolayers with 0 5% sodium dodecyl sulphate (SDS) for 10 min and adding the cpm in the SDS lysate to the spontaneous release Monocyte mediated target cell cytotoxicity is expressed as percentage specific lysis (% SL)

$$\% SL = \frac{\text{supernatant cpm (monocytes + NHIK 3025)} - \text{supernatant cpm (NHIK 3025)}}{\text{Total incorporated cpm}}$$

Total incorporated cpm was 14007 ± 3759 (n = 6) Controls of target cells with LPS were included in all experiments and % SL in monocyte/target cell cocultures with LPS was calculated using the spontaneous release in these controls LPS did not influence significantly the spontaneous release from target cells in the absence of monocytes (24 6 ± 1 2% of total incorporated cpm in target cells in HS M 23 8 ± 1 4% target cells with LPS 0 1 ug/ml assayed after 48 hours n = 6)

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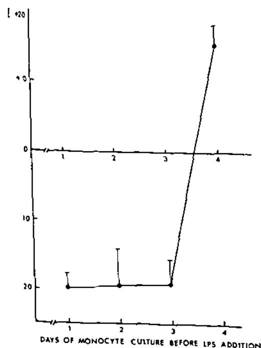


Fig 1 Influence of addition of LPS to monocyte cultures at various times before or simultaneous with addition of target cells Target cells were added to all cultures on day 4 Treatment induced change in cytostatic activity is expressed as cytostatic index (CI) Percentage target cell inhibition of normal monocytes 12 7 ± 7 8 Mean ± S E M n = 4

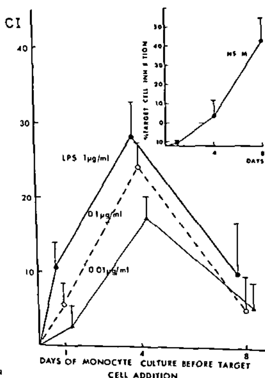


Fig 2 Cytostatic activity (CI) induced by addition of LPS simultaneous with target cells to monocytes cultured for various times ▲—▲ LPS 0 01 µg/ml O---O LPS 0 1 µg/ml ●—● LPS 1 µg/ml Percentage target cell inhibition of normal monocytes in the same experiments is shown in the insert Mean + S E M n = 6

sensitive to LPS enhancement, both in terms of CI and in the absolute reduction of target cell cpm induced by LPS The differentiation-related cytostatic activity of untreated monocytes (Fig 2, insert) did not correlate with the responsiveness to LPS enhancement

Effect of LPS on the Cytostatic Activity of Lymphokine-activated Monocytes

Preincubation for 24 hours in lymphokine-rich supernatants of *C parvum*-stimulated lymphocytes induces cytostatic ability in monocytes when the monocytes are challenged with target cells immediately after lymphokine exposure (8) When LPS was present during monocyte/target cell interaction, the cytostatic influence of both lymphokine-treated (MAF) and normal monocytes was significantly ($p < 0.008$) enhanced (Fig 3) The LPS-induced enhancement was of about the same order of magnitude in lymphokine-treated and normal monocytes

Monocytes activated by a 24-hour exposure to lymphokines were cultured for 48 hours in HS M

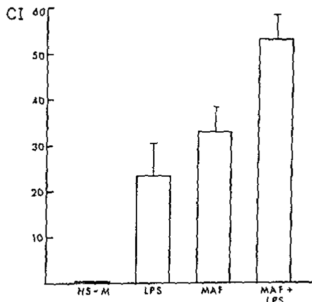


Fig 3 LPS and lymphokine (MAF) enhancement of monocyte-mediated cytostatic activity expressed as CI Cultures indicated by MAF were incubated with lymphokine supernatants from day 3 to day 4, the remaining cultures receiving fresh HS-M Target cells were added on day 4 cultures indicated by LPS receiving LPS 0 1 µg/ml at the same time Percentage target cell inhibition of normal monocytes (HS M) −0 1 ± 7 5 Mean ± S E M n = 8

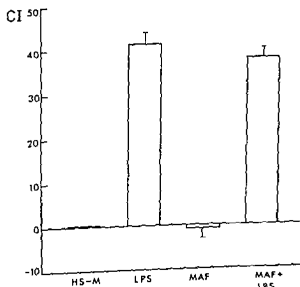


Fig 4 Cytostatic activity (CI) induced by LPS in monocytes previously exposed to lymphokines and deactivated by subsequent *in vitro* culture Cultures indicated by MAF were incubated with lymphokine supernatants from day 3 to day 4, the remainder receiving fresh HS-M All cultures received fresh HS M on day 4 and after 48 hours of further culture target cells were added on day 6 LPS 0 1 µg/ml was added together with target cells to cultures indicated by LPS Percentage target cell inhibition of normal monocytes 24 7 ± 18 5 Mean ± S E M n = 4

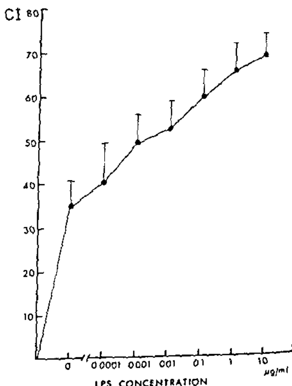


Fig 5 Cytostatic activity (CI) induced by different concentrations of LPS added to lymphokine activated monocytes Lymphokine supernatants were added from day 3 to day 4 of monocyte culture Target cells and LPS were added on day 4 Percentage target cell inhibition of normal monocytes −0 8 ± 8 8 Mean ± S E M n = 6

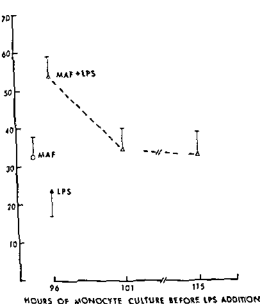


Fig 6 Influence of LPS addition at various times during monocyte/target cell interaction LPS to a final concentration of 0 1 μg/ml was added to lymphokine treated monocytes (△- -△) simultaneously with the target cells (96 hours) 5 hours later (101 hours) or 19 hours later together with the methyl ³H TdR pulse (115 hours) Cytostatic index (CI) of monocytes receiving only lymphokine supernatants from day 3 to day 4 (O MAF) and monocytes receiving only LPS together with the target cells (▲ LPS) is also shown Percentage target cell inhibition of normal monocytes −0 1 ± 7 5 Mean ± S E M n − 8

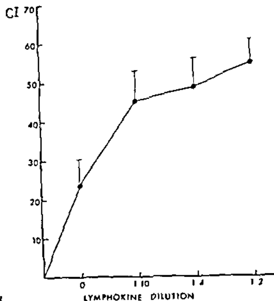


Fig 7 Cytostatic activity (CI) induced by various dilutions of lymphokine supernatants assayed with LPS 0 1 μg/ml present in the monocyte/target cell coculture Monocytes were incubated in lymphokine dilutions from day 3 to day 4 and target cells together with LPS added on day 4 Percentage target cell inhibition of normal monocytes −0 2 ± 8 8 Mean ± S E M n 7

leading to reversal of the lymphokine induced activation (8) and then assayed for cytostatic activity in the presence or absence of LPS (Fig 4) However the LPS response was not larger in the monocytes previously exposed to lymphokines than in control cells

The LPS induced response in lymphokine treated cells was dose-dependent (Fig 5) significant ($p < 0.05$) enhancement being observed at doses as low as 0 1 ng/ml By adding LPS at different times during the coculture of lymphokine-activated monocytes and target cells (Fig 6) it was apparent that the LPS response was abrogated if LPS was present only during parts of the monocyte/target cell interaction

A dose response relationship between lymphokine dilution and monocyte response could be established even when monocyte mediated cytostasis was assayed with LPS present during the assay (Fig 7)

Cytostatic Effect of Soluble Factors Released from Lymphokine activated Monocytes Exposed to LPS

Monocyte supernatants obtained from monocytes cultured for 24 hours after lymphokine activation in the presence or absence of LPS were tested for immediate (5 hours) or delayed (29 hours) influence on DNA synthesis in target cells plated alone The monocyte supernatants were diluted 1 2 with fresh HS M to avoid interfering effects of nutritient depletion

No inhibitory activity was found in the supernatants of normal monocytes lymphokine activated monocytes or monocytes treated only with LPS either on short or long term exposure (Fig 8) However the supernatants of monocytes first activated with lymphokines and subsequently exposed to LPS contained suppressive activity on target cell methyl ³H TdR incorporation The effect was not detectable at 5 hours but was consistent in all experiments at 29 hours ($p < 0.016$) The active supernatants contain LPS and the effect might thus be due to an LPS effect on the target cells rendering them susceptible to supernatant factors from lymphokine activated monocytes This possibility was tested by exposing target cells to supernatants

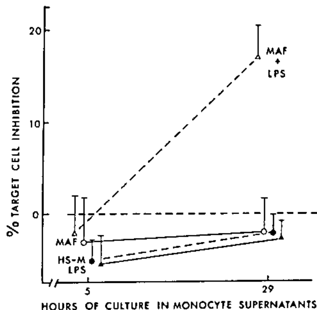


Fig 8 Cytostatic activity of monocyte supernatants expressed as percentage target cell inhibition Monocyte cultures indicated by MAF were incubated with lympho kine supernatants from day 3 to day 4 the remainder receiving HS M On day 4 LPS 0 1 µg/ml was added to monocyte cultures indicated by LPS the remainder receiving HS M The monocyte supernatants were harvested on day 5 diluted 1 2 with fresh HS M and added to target cell cultures plated 24 hours earlier One µCi methyl ³H TdR was added together with the monocyte supernatants (5 hours) or 23 hours later (29 hours) and the target cell cultures were harvested after 5 hours of methyl ³H TdR incorporation Target cell methyl ³H TdR incorporation in the medium control 62000 ± 5400 cpm (5 hours) and 122 600 ± 6 800 cpm (29 hours) △----△ MAF + LPS ○—○ MAF ●---● HSM ▲—▲ LPS Mean ± S E M n = 6

from activated monocytes with LPS present only during the assay Only supernatants from activated monocytes subsequently exposed to LPS contained inhibitory activity (Table 1) LPS presence or absence in the assay being of no consequence

Influence of Heat Inactivation of Serum on LPS Enhancement of Cytostasis

The experiments were repeated at least twice with medium supplemented with heat inactivated (56 °C 30 min) human AB serum during all stages where LPS was present in the medium The results were almost identical with those presented (data not shown)

Effect of LPS on the Cytolytic Ability of Normal and Lymphokine-activated Monocytes

The effect of LPS on monocyte mediated target cell lysis was examined in an assay measuring release of methyl ³H-TdR from prelabelled target cells Lymphokine activation of the monocytes prior to addition of the target cells enhanced significantly the specific lysis observed after 48 hours ($p <$ 0 016) (Fig 9) Addition of LPS during the assay period did not influence the spontaneous release of methyl ³H-TdR from target cells but enhanced the cytotoxic activity of both previously untreated and lymphokine activated monocytes ($p \leq$ 0 031)

The kinetics of monocyte mediated lysis in an experiment displaying a relatively high level of lymphokine activation are shown in Fig 10 LPS and lymphokine enhancement are greatest after 48 hours The increase in cytolytic activity in untreated cultures is greatest between 48 and 72 hours while

TABLE 1 *Cytostatic Factor Activity is not Due to LPS Damage to Target Cells*

Monocyte treatment 3rd–4th day	Monocyte treatment 4th–5th day	LPS addition to target cells in assay	% inhibition of target cell methyl ³H TdR incorporation compared with medium control
HS M	HS M	0	−1 2 ± 2 6
HS M	HS M	LPS	−4 3 ± 1 9
HS M	LPS	0	5 4 ± 1 3
MAF	HS M	0	2 3 ± 0 2
MAF	HS M	LPS	−2 4 ± 2 4
MAF	LPS	0	23 5 ± 2 4
–	–	0	0
–	–	LPS	−1 3 ± 0 9

Monocytes were cultured for 3 days before addition of CP induced lymphokine supernatants (MAF) On day 4 the ... monocyte cultures and fresh HS M with or without LPS was added On day 5 the ...

One µCi methyl ³H TdR/culture was ...
supernatants Mean ± SD of triplicate cultures

the increase in lymphokine treated cultures decreases at the same time

If observed for 72 hours or longer, the isotope release in untreated cultures in some experiments rose to higher levels than in lymphokine or LPS treated cultures and a reduction in adherent effector cells in lymphokine and LPS treated cultures was sometimes observed microscopically

DISCUSSION

Data concerning the conditions which modulate target cell inhibition or killing by human mononuclear phagocytes in the absence of antibody are very scarce (6 12) Previous work in this laboratory has demonstrated that human monocytes (23) and peritoneal macrophages (7) acquire cytostatic ability on *in vitro* differentiation and that additional cytostatic activity can be induced by lymphokines at all *in vitro* differentiation stages (8)

The present study supports the view based on data from animals models viz that LPS may act as an expression signal of mononuclear phagocyte mediated tumour cell inhibition (10 18, 19) Untreated monocytes seem to be most sensitive to LPS enhancement at an intermediate stage of *in vitro* differentiation from monocytes to the large macrophage like cells found after seven days *in vitro* Increased LPS responsiveness of mononuclear phagocytes at certain stages of functional maturation has been demonstrated repeatedly in animal systems (10 18 19 21) LPS responsiveness in animal macrophages usually requires prior stimulation or activation with stimuli such as *in vivo* peptone or BCG injection (21), although there are reports to the contrary (17) No additional LPS responsiveness seems to be induced by *in vitro* lymphokine exposure in our system in terms of monocyte cytostatic activity This applies regardless of whether the monocytes are exposed to LPS at the time of maximal lymphokine induced cytostatic activity or following deactivation by *in vitro* culture

The impressive structural and functional changes observed in untreated cultures (7 23) demonstrate clearly that *in vitro* culture in this system is a strong signal by itself for monocyte differentiation and may well provide the necessary »priming« stimulation for LPS responsiveness The finding that dose/response curves can be established for both LPS and lymphokines in cultures treated with a fixed concentration of the other agent suggests a true pharmacological effect of both agents

The activation of human monocyte mediated tumour cell cytolysis by lymphokines has been

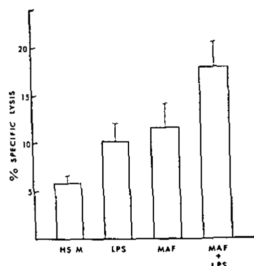


Fig 9 LPS and lymphokine enhancement of monocyte mediated cytolysis expressed as percentage specific lysis of target cells prelabelled with methyl ^{3}H TdR Monocyte cultures indicated by MAF were incubated with lymphokine supernatants from day 3 to day 4 the remainder receiving HS M Prelabelled target cells were added to the monocyte monolayers on day 4 cultures indicated by LPS receiving LPS 0 1 µg/ml at the same time Release of methyl ^{3}H TdR was assayed after 48 hours of monocyte/target cell interaction Mean ± S E M n = 6

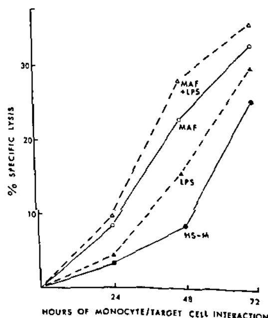


Fig 10 Kinetics of monocyte mediated cytotoxicity to target cells prelabelled with methyl ^{3}H TdR Monocyte treatment as in the text to Fig 9 ●——● HSM ▲---▲ LPS ○——○ MAF △---△ MAF + LPS Mean of triplicate cultures Standard deviation (SD) less than 10 % of the mean

described once previously (6) while we have been unable to find reports concerning the effect of LPS on the expression of cytotoxicity The long assay period required for demonstration of monocyte-mediated target cell lysis makes interpretation of the results difficult, since important modifications of monocyte function, such as culture-induced differentiation (7) and reversal of lymphokine activation (8) are known to occur during this phase of culture The kinetics of the response could be compatible with the induction of functionally relatively short-lived cytotoxic effector cells by lymphokines and LPS, in accordance with data obtained in animal systems (17, 18) The observation that untreated cultures may develop higher levels of target cell lysis on prolonged (> 72 h) interaction with target cells may be a reflection of the impaired culture-induced differentiation found after LPS (9) or lymphokine (24) exposure

In the previous article (9), depressive effects of LPS on mononuclear phagocyte protein synthesis, phagocytosis, degradation of ingested material and induction of cytostatic activity were observed in the same system and with similar LPS concentrations as those employed for the demonstration of cytostatic and cytotoxic enhancement This questions the view that LPS enhancement occurs at low doses and LPS toxicity at high doses (20) Recently, *Rosenstreich et al* demonstrated simultaneous LPS toxicity and stimulation in terms of soluble factor production in mouse macrophages (16) The question as to whether LPS toxicity and stimulation are causally linked in some way has not been satisfactorily answered We have demonstrated earlier that lymphokine-activated monocytes contain intracellular substances capable of inhibiting target cell proliferation (8) The soluble cytostatic factor released from lymphokine treated monocytes exposed to LPS demonstrated in this study may be related to the cytostatic factors found intracellularly before exposure to LPS The effect of LPS on monocyte membrane structure and function indica-

is not synonymous with monocyte death since there was no change in adherent cell numbers and numbers of cells taking up trypan blue in cultures producing active supernatants after removal of the supernatant However, after one week of further culture the number of monocytes in such cultures was clearly reduced, thus indicating a shortened life span of cells treated in this way

Release of several soluble factors from animal ... incor- ... A. ... 4. ... or actually lyse target cells has been ...

15) The soluble factor is probably not cold thymidine (15), since no inhibition of target cell ³H methyl-TdR-incorporation is found after five hours of exposure to the factor The kinetics of the inhibition, with a delayed effect first apparent after 29 hours, resembles the *in vitro* action of tumour necrosis factor (TNF) a cytostatic glycoprotein of probable macrophage origin found in the serum of mice pretreated with *C Parvum* and subsequently exposed to LPS (11)

Control experiments with heat-inactivated serum indicate that the cytostatic enhancement and induction of cytostatic factor release produced by LPS are not dependent on heat labile serum derived complement components

Studies of the *in vitro* conditions modulating the induction and expression of human mononuclear phagocyte cytotoxicity to tumour cells may provide means of manipulating these cells to increased participation in the *in vivo* eradication of human tumour cells

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